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VOL. V. No. 1] [FEBRUARY, 1924.

THE BRITISH JOURNAL OF EXPERIMENTAL PATHOLOGY

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LONDON: H. K. LEWIS & CO. LTD., 136, GOWER STREET, W.C. 1.
EDITORIAL AND PUBLISHING OFFICE: 28, GOWER PLACE, W.C. 1.
CANADA: THE MACMILLAN COMPANY OF CANADA, LTD., BOND STREET, TORONTO.

Annual Subscription, January to December, post free, Two Pounds net.
Single Numbers, Seven and Sixpence net.

THE BRITISH JOURNAL OF EXPERIMENTAL PATHOLOGY.

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THE EDITORS,

British Journal of Experimental Pathology,

28, GOWER PLACE,

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THE BRITISH JOURNAL

OF

EXPERIMENTAL PATHOLOGY

EXPERIMENTS ON IMMUNITY TO TUMOUR GROWTH.

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Received for publication December 31st, 1923.

THE subject of tumour immunity has for a long time been looked upon as an unexplained problem of a different nature from immunity reactions in general, for all efforts to trace a relationship between the two conditions have been unsuccessful. This failure to establish a connection may be due to the difficulties of the subject, and it is possible that as knowledge increases the phenomena of tumour immunity will be found to fall into line with those of immunity to bacterial diseases. Clearly much progress is needed before a wide generalization of this kind can be placed on a substantial basis. The following paper records certain experimental results which point in this direction, and indicate that, in the case of Jensen's rat sarcoma, tumour immunity is a response of the animal to the absorption of a specific foreign proteid.

The experiments described in this paper are a continuation of certain work done by us in collaboration with Prof. Russ during the past few years on the immunization of animals with irradiated tumour material. Work on this subject was first done by Contamin (1910[1 and 2]). It has since been investigated in the Cancer Research Laboratories of the Middlesex Hospital by Russ, conjointly with Wedd and Morson (1914), with Mottram (1917), and with Chambers and Scott (1922).

Since the early days of the work on transplanted tumours it has been known that if an animal is inoculated with living tumour the greater part of the inoculated mass rapidly dies and becomes autolyzed, in fact, the whole of the material undergoes this change except the part in close contact with the vascularized tissues around the site of inoculation. Owing to this well-known fact much attention has been given to testing the therapeutic value of extracts of autolyzed tumour. The earliest work of this kind was done by Jensen (1903, 1909), and Von Leyden and Blumenthal (1902); papers on the subject have been published by Abderhalden (1914), Blumenthal (1914), Lewin (1912),

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Bosch (1919), Nather (1922), and many others. On the whole, the results have been very uncertain and contradictory, and no good evidence has yet been produced showing that autolytic extracts can cause immunity.

On the other hand, it has long been known that the treatment of animals with tissue emulsions may result in stimulation of tumour growth. Bashford, Murray and Haaland (1908) recorded that the treatment of mice with mamma emulsion increased their sensibility to subsequent inoculations with squamous-celled carcinoma; they obtained similar results by treatment with emulsions of embryo. Haaland (1910) showed that the mechanical disintegration of tissue not only resulted in the loss of all its immunizing power, but that animals treated with this material subsequently grew tumours larger than the controls. Carrel (1913) showed that when living tissues were grown *in vitro* the addition of extracts prepared from other tissues enormously increased the growth. His conclusions have been recently extended by Drew (1922, 1923).

Our experiments have yielded very similar results; in fact, it has been common to find that the treatment of rats with extracts prepared from tumour tissue (J.R.S.) has caused them to grow larger tumours than the untreated animals.

Much evidence is therefore available indicating that autolyzed tissue contains some substance able to stimulate tumour growth. The nature of this body and the conditions under which it is formed, absorbed and excreted, are questions of great importance in the study of malignant disease; still more so if we attempt to trace the reason why the opposite effect is so often produced with transplanted tumours, and immunity obtained. It is easy to conceive that the existence of this stimulating body may mask an antigen should this be present at the same time.

It is necessary to explain here the working hypothesis for the experiments described in this paper.

THE WORKING HYPOTHESIS.

During the last few years the writers have made repeated attempts to extract an antigen, from both irradiated and non-irradiated tumour (J.R.S.), capable of causing immunity (Chambers, Scott and Russ, 1922), but without success. One of us (H. C.) came to the conclusion some time ago that the failures were easily explained if the active agent was not present at the time the tissue was used, but was formed some time later from some precursory body contained in the tumour-cells. An experiment has been described elsewhere (Chambers, 1924) which suggested that if a tumour established in an animal was irradiated, the antigen was gradually absorbed for several days after the treatment had been given, but was not absorbed at once. In this experiment small tumours were completely excised twenty-four hours or three days after they had been exposed *in vivo* to a lethal dose of radiation. A test inoculation of these animals showed that the degree of protection increased the longer the irradiated tissue remained in the body; the immunity was only slight after twenty-four hours. It was then considered probable that the tumour-cells, though injured, must be alive to produce the antigen, and that this substance was formed during death.

Much evidence has now accumulated showing that tumour-cells which are

actively growing and healthy are unable to create any defensive reaction in the animal in which they are established. This fact is obvious in the case of human cancer and is also true for Jensen's rat sarcoma, although this latter tumour is able under special conditions to create a high degree of immunity. It has seemed to us that this observation is of fundamental importance, for it furnishes a possible explanation of the differences of behaviour of spontaneous and transplanted cancer animals, and supplies a working hypothesis for further investigations.

Clearly, if actively-growing tumour-cells are unable to create a defensive reaction, we have to assume that degenerate cells produce some substance not normally excreted by healthy ones, or look for some other explanation.

It seemed very improbable that, in producing immunity, some entirely abnormal body should be formed by degenerate tumour-cells, and much more likely that during the profound changes brought about by autolysis after death certain bodies were formed, the ultimate fate of which depended upon the local conditions around the dead cells. It was easy to conceive that such variable factors as local vascular supply, the rate of autolysis, the relative diffusibility of the products of autolysis and the ease with which these substances might be destroyed by enzyme action could affect the nature of the bodies absorbed into the general system of the animal, and in this way modify the result obtained. With this outlook it was necessary to trace the various stages of autolysis and determine the biological properties of the proteids given off at different times, especially the ability of these proteids to produce immunity.

Experimental results have given indications of the production of two bodies from tumour tissue which have diametrically opposite biological effects. One of these has been known for the last fifteen years, though our knowledge of its properties and conditions of absorption and excretion are most meagre. It is easy to detect, apparently resists enzyme action, and causes stimulation of tumour growth. The other creates immunity, and until now has not been separated from the living cell.

Our earliest experiments were carried out on the hypothesis that the antigen was produced by ferment action, but was probably unstable and easily destroyed by continued ferment action.

It also seemed essential that the separation of the stimulating body from the antigen should be attempted, if consistent results were to be obtained. For this purpose two methods of investigation were available; the first relied on a possible difference in the rate of production of the two bodies, the second on the possibility that they were proteids of different molecular size, and could be separated by some of the methods in use for the fractional separation of proteids.

THE EXPERIMENTAL INVESTIGATION.

The Technique.

The tumour.—The tumour used for this work, *i. e.* Jensen's rat sarcoma, gives nearly 100 per cent. of successful inoculations, but often produces concomitant immunity. It has already been shown (Chambers, 1922) that, if healthy cells from this tumour are inoculated into a series of normal rats

comparatively little concomitant immunity is obtained, but if cells which have previously undergone partial degeneration are inoculated a high degree of protection occurs. For this reason, and because these tumours are rarely homogeneous in structure, and nearly always show signs of central degeneration, the technique employed is an all-important factor. On account of the degenerative changes, it is often difficult, if not impossible, to be sure that all the animals of a series receive exactly similar treatment. This difficulty can only be overcome by using meticulous care in the choice of material. Tumours which have grown rapidly, or recurrent growths, have been specially selected, and only tissue at the growing edge used. With these conditions maintained the control animals of many successive series have given results which are fairly comparable with each other. It is obvious, however, that, notwithstanding care in technique, some variation occurs, and therefore a large number of animals have had to be used.

The diluting fluid.—It was necessary to find some fluid which could be mixed with the tumour-cells and which would be unlikely to affect the proteids; the formula given by Drew (1922) for the growth of tissues *in vitro* seemed to be the most suitable, and Drew's fluid has been used in all these experiments.

Shaking apparatus.—It was necessary that the mixture of tumour-cells and fluid should be kept constantly shaken and the temperature controlled. This has been done by putting a flask containing the material inside an incubator in a box attached to a water-wheel arranged to shake 5 to 10 times a minute. We are indebted to Dr. J. N. Goldsmith, F.I.C., for the design of this effective contrivance.

Radiation.—Whenever radiation has been used, the tumours have been excised with aseptic precautions, minced into a shallow dish, covered with a sheet of mica, and exposed to X rays from a Coolidge tube working under standard conditions (Chambers, Scott and Russ, 1922). The whole of the material has been given a lethal dose.

The Early Stages of Autolysis.

Experiment 1.—A preliminary experiment was done in which 10 c.c. of minced irradiated tumour (J.R.S.) were shaken with 50 c.c. of Drew's fluid at 37° C., a few drops of toluol being added to prevent bacterial growth. After 21 hours' incubation the mixture was centrifugalized in a high-speed centrifuge, and 20 rats were inoculated in the left flank with 0·5 c.c. of the fluid, which was turbid. A second series of 20 rats were inoculated with 0·2 c.c. of the cell deposit. Four weeks later all the experimental animals, together with 20 controls, received a test inoculation of tumour into the right flank. Following the usual laboratory technique, the tumours which subsequently formed were measured twice a week by means of a pair of dividers and a millimetre scale. The results showed a reduction in the size of the tumours in both experimental series amounting to approximately 25 per cent. of the average volume of the controls. These results, though exhibiting too small an effect to be convincing, led to further experiments in which the time of autolysis was reduced.

TABLE I.

		No. of rats.	Days after inoculation. 11	14	18	21	25	28	32
Average volume of tumours in c.c.	B. Experimentals	14	0·11	0·38	0·80	1·23	3·05	3·61	4·38
	A. Controls	20	0·54	1·15	3·55	7·21	13·7	22·8	34·3

Experiment 2.—The tumour was removed and irradiated and kept at a room temperature overnight. 10 c.c. of this (J.R.S.) were shaken with 50 c.c. of Drew's fluid for 6½ hours at 42° C., a few drops of toluol having been added. The mixture after incubation was centrifugalized, and 0·5 c.c. of the turbid fluid inoculated subcutaneously into both flanks of 20 normal rats. 4 weeks later the 16 surviving animals (apparently in good health) received a test inoculation together with 20 controls. The results are shown in Fig. 1, the tumours being drawn to scale and each horizontal line representing the growth of a tumour in one rat. It will be seen that the tumours in the experimental animals are much smaller than those in the controls. Table I shows the average volumes of the tumours in the two series, reaching 4·38 c.c. in the treated animals compared with 34·3 c.c. in the untreated. These results were so unusual and striking that further experiments were put in hand for confirmation.

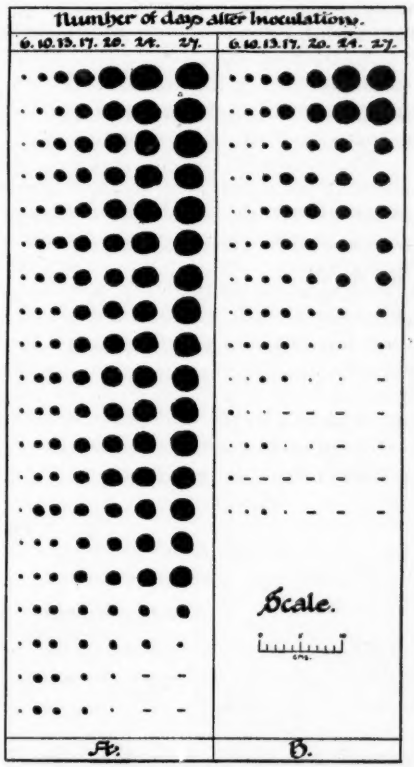

FIG. 1.
A. CONTROLS.
B. EXPERIMENTALS.

Experiment 3.—The next series was carried out on similar lines, the mixture being incubated for 6 hours at 42° C. Several alterations were made in the technique, thinking that they would be an advantage. The amount of tumour was reduced, 10 c.c. being added to 100 c.c. of Drew's fluid; the animals were given a second treatment, and the test inoculations were done after a fortnight instead of waiting 4 weeks. The results failed to show any immunity in the treated animals. In view of this discrepancy it was next decided to track each variation of the technique in turn.

Experiment 4.—Experiment 3 was repeated as closely as possible using

the same amount of tumour as before, viz. 10 c.c. to 50 c.c. of Drew's fluid. Again the results failed to show any immunity in the treated animals.

Experiment 5.—Experiment 3 was done again, but in this case 3 series of 20 rats were inoculated in two sites with 1·0 c.c. of the fluid after different periods of autolysis at 42° C., viz. after 2 hours, 4 hours and 6 hours respectively. 9 days later the treatment was repeated with freshly prepared autolytic fluid.

An epidemic unfortunately attacked the animals of the 6-hour group and reduced their numbers so that this part of the experiment was spoilt. The animals of the other two series remained well, and two weeks after the first treatment they received a test inoculation together with 20 controls. The results are shown in Table II. It will be seen that there is not much effect, though the difference between group B and the controls is greater than that usually obtained with successive series of controls.

Experiments 3 to 5 all differed from the first two (1 and 2) in that the time between the treatment of the animals and the test inoculations was shorter, the interval being two weeks instead of four. No repeat experiments have yet been done with longer time interval. The reason for this has been chiefly because in the meanwhile other tests had been in progress which tended to show that even during the early stages of autolysis the stimulating body was formed. It appeared in all these experiments, where the whole of the fluid was inoculated, that we were introducing stimulating substance in addition to antigen if the latter was present at all. With a complete lack of knowledge of the effect of a mixture of these bodies, and of the rate of excretion of the stimulating substance, we were not likely to obtain consistent results by continuing on these lines. Separation of the two bodies seemed, therefore, essential to success.

TABLE II.

		No. of rats.	Days after inoculation. 6	9	13	16	21	24
Average volume of tumours in c.c.	B. Experimentals (2 hours' incubation)	20	0·17	0·40	1·50	2·92	6·00	12·7
	C. Experimentals (4 hours' incubation)	20	0·20	0·59	2·51	4·57	12·4	22·4
	A. Controls	20	0·22	0·53	2·12	4·95	11·2	17·6

The suggestion is made that the discrepancy between the results of Experiments 1 and 2 and those of Experiments 3, 4 and 5 is perhaps due to the different interval between the treatment and the test inoculations, and in the case of the longer interval to the animals having succeeded in separating the antigen from the stimulating body by eliminating the latter. The effects of antigen are known to be lasting; there is evidence also that the stimulating body affects the growth of tumours for some weeks, though for how long is a matter requiring investigation. Reference to Table II suggests a slight immunizing effect with the 2-hour fluid but stimulation with the 4-hour—a result which could easily be understood if the two bodies do not appear simultaneously.

Acetone Precipitation of the Stimulating Substance.

It has already been shown that stimulation of tumour growth occurs when tumour-cells which rapidly autolyse are inoculated, *i. e.* when they have been mechanically disintegrated; free contact between the autolyzed material and the living tissues of the animal around the site of inoculation has apparently been established, allowing free absorption of the products of autolysis. On the other hand, if a piece of intact tumour is inserted, similar autolytic changes take place in the centre of the inoculated mass, and yet it quite frequently happens that neither stimulating substance nor antigen reaches the general system of the animal. Inflammatory reaction has apparently shut off the zone of autolysis fairly completely.

Between these two conditions, the one isolating the autolyzed tissue and the other allowing free absorption, there seems to be an intermediate stage in which the animal succeeds in absorbing antigen and leaving stimulating substance behind. This possible explanation has led us to think that the two bodies may be of different molecular size, the antigen being the smaller of the two and more readily absorbed. If this theory is true it may be possible, by making use of dehydrating agents such as alcohol or acetone, to separate the one body from the other. Large proteid molecules are mechanically precipitated by lower concentrations of a dehydrating agent than are smaller ones, and the larger can be de-natured or rendered insoluble by exposure to strengths of alcohol, which do not have so much effect on the smaller molecules. On this working hypothesis experiments were put in hand. Acetone was used rather than alcohol as the dehydrating agent because of the lower boiling-point of the former, and the greater ease with which acetone could afterwards be eliminated if necessary.

In the many attempts we have made to extract the antigen, irradiated, non-irradiated, degenerate or non-degenerate tumour (J.R.S.) has been rubbed down with various diluting fluids, including normal rats' serum, saline and Locke's fluid. The result of inoculating the fluid extracts has always been that we have failed to produce immunity. It seemed probable, therefore, that the antigen must be derived from the constituents of the cell which are not normally soluble in either serum or saline, probably from the nuclear structures. Many experiments have been done using the residue left after the soluble constituents of the cell have been washed away. The following is one of them:

Experiment 6.—20 c.c. of minced tumour (J.R.S.) were rubbed down in a mortar and washed many times with Drew's fluid, 600 c.c. being used. The insoluble cell residue was put into a flask with 200 c.c. of Drew's fluid, a little toluol added, and the mixture shaken at 42° C. for 4 hours. The fluid was then filtered off through glass wool and mixed with acetone to a strength of 50 per cent., the resulting precipitate being collected on a filter-paper. The acetone was drained away and the precipitate washed many times with 14 c.c. of sterile normal saline. A clear colourless fluid was thus obtained which gave the Biuret reaction and became slightly turbid on boiling. ½ c.c. of this fluid was inoculated subcutaneously into the left flank of 20 normal rats; no local reaction occurred. Four days later the animals were

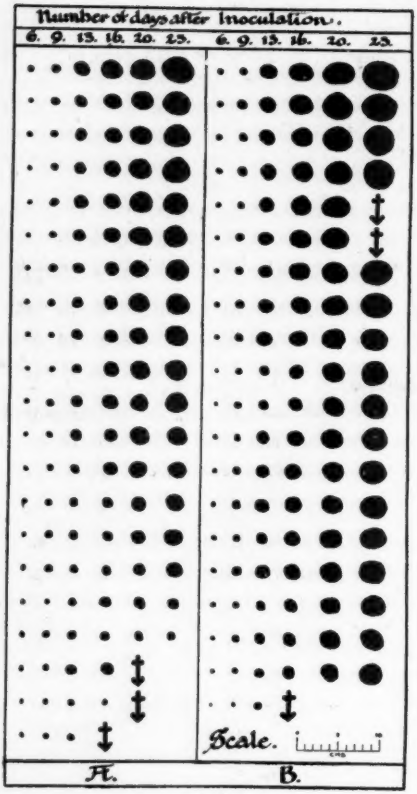

FIG. 2.
A. CONTROLS.
B. EXPERIMENTALS

given a repeat treatment with fresh extract prepared in the same way. Eleven days after the first treatment all the experimental rats and 20 controls received a test inoculation into the right flank. The results are given in Fig. 2 and Table III.

It will be seen that the tumours in the experimental series are much larger than those in the controls, the average volume in the treated animals reaching 34·4 c.c., compared with 14·6 c.c. in the untreated. The tumours in the treated animals grew so rapidly that the experiment was brought to an end much sooner than usual, in fact in this group all the animals had to be either killed or operated upon. In our experience it is unknown with this type of tumour, to have every animal of the control series growing progressively, for two or three in every group of twenty invariably retrogress. Clearly stimulation had been produced.

The results are confirmatory of the theory that the stimulating body as well as the antigen is derived from the nuclear constituents of the cell. They also indicate the formation of this substance in the early stages of autolysis, and show that precipitation of the proteids with 50 per cent. acetone carries down the stimulating body as well. Clearly it is not rapidly eliminated when once absorbed into the general system of the animal.

TABLE III.

		No. of rats.	Days after inoculation. 6	9	13	16	20	23
Average volume of tumours in c.c.	B. Experimentals	20	0·10	0·48	2·98	8·33	18·8	34·4
	A. Controls	21	0·07	0·27	1·26	3·48	8·46	14·6

The Stimulating Substance does not overcome an Established Immunity.

Experiment 7.—Fifty-four immune rats were given a test inoculation of Jensen's rat sarcoma. Two weeks later the animals were divided into two groups, and selected so that the two series were as far as possible the same. The animals of one group were treated with extract prepared as in

Experiment 6, a second treatment being given 7 days later. Three days after the first treatment all the animals received a test inoculation. No difference could be detected in the two series; the tumours failed to grow in both, though large tumours formed in the normal control rats inoculated at the same time.

Acetone Precipitation of the Antigen.

Experiment 8.—10 c.c. of minced tumour (J.R.S.) were shaken in 100 c.c. of Drew's fluid with a few drops of toluol, at 36° C. for 20 hours. The mixture was then centrifugalized, the fluid separated and acetone added to it until a strength of 80 per cent. was reached. The heavy precipitate which formed was allowed to stand overnight and was then collected by centrifugalization, the acetone drained away as completely as possible and the precipitate shaken repeatedly with an equal volume of sterile normal saline. This mixture was centrifuged, and 1 c.c. of the clear fluid was inoculated subcutaneously into the left flank of 18 normal rats. Eighteen days later all the treated animals and 20 controls received a test inoculation into the right flank.

The washings from the precipitate in normal saline gave a biuret reaction and a slight coagulum on boiling. Where the fluid was injected all the animals showed a small inflammatory reaction, and in a few the skin gave way. Under the conditions of the experiment the fluids would contain cell enzymes and these would account for the local effect.

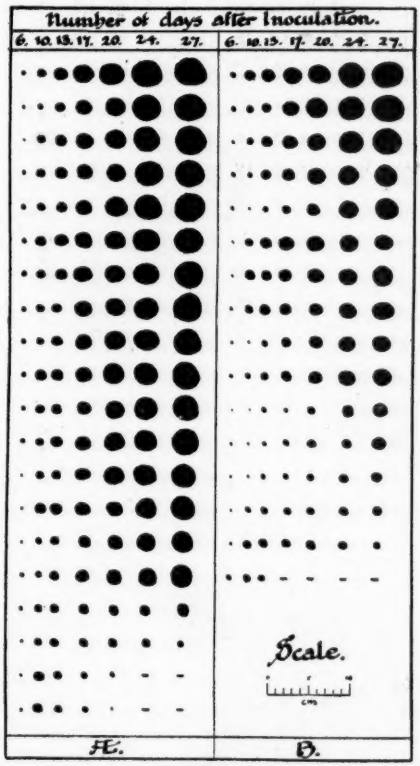

FIG. 3.
A. CONTROLS.
B. EXPERIMENTALS.
THE CONTROL ANIMALS IN THIS FIGURE ARE THE SAME AS THOSE IN FIG. 1.

The results of the test inoculation are shown in Fig. 3 and Table IV. It will be seen that the tumours in the experimental animals are smaller than those in the controls, the average volume in the treated animals reaching 12·9 c.c. compared with 34·3 c.c. in the untreated animals.

TABLE IV.

		No. of rats.	Days after inoculation.						
			11	14	18	21	25	28	32
Average volume of tumours in c.c.	B. Experimentals	17	0·27	0·60	1·28	2·71	5·67	8·95	12·9
	A. Controls	20	0·54	1·15	3·55	7·21	13·7	22·8	34·3

Experiment 9.—An experiment was done using fractional precipitation with 0 to 50 per cent. acetone and 50 to 80 per cent. acetone to see if stimulating substance together with the cell enzymes could be carried down with the lower concentration of acetone and antigen left in the fluid, the latter being subsequently precipitated with the higher concentration.

20 c.c. of minced tumour (J.R.S.) were shaken for 18 hours at 36° C. with 100 c.c. of Drew's fluid. The fluid was filtered off through glass wool and acetone added to 50 per cent. The precipitate which formed was removed by filtration through filter-paper and more acetone added to the clear filtrate to a strength of 80 per cent. A second precipitate was thus obtained, which was again filtered off; this was dissolved completely in 8 c.c. of sterile normal saline, and 0·3 c.c. of the solution inoculated into 20 normal rats in two sites. No local reaction occurred. Fourteen days later all the experimental animals and 20 controls received a test inoculation into the right flank. The results are shown in Table V. It will be seen that the average volume of the tumours in the treated animals reached 13·3 c.c. as compared with 26·7 in the untreated.

TABLE V.

		No. of rats.	Days after inoculation.					
			7	11	14	18	21	26
Average volume of tumours in c.c.	B. Experimentals	19	0·17	0·84	2·18	4·42	7·36	13·3
	A. Controls	20	0·21	1·18	3·09	7·43	14·3	26·7

This experiment has been repeated for shorter periods of autolysis, *i.e.* 2 hours; it has been done after autolysing for 3 and 6 days. The results showed no immunity.

The Antigen Value of Tumour-cells after Treatment with Formalin.

In the experiments described in the previous section we have attempted to obtain the antigen by making use of the normal intracellular enzymes to digest the cell structures. This method has many disadvantages, the chief being that we are unable to control the process completely, or to produce conditions comparable with what occurs in an animal after an inoculation. It seemed worth while therefore to test whether the enzymes for digesting the tissue could be supplied by the animal into which the inoculation was made. For this purpose it was necessary to insert the nuclear structure of the cells after having destroyed the intra-cellular enzymes by the action of some agent which would not destroy the antigen. Formalin is known to do this in the case of bacteria. The following experiment was therefore done on similar lines to the method used by Dreyer (1923) for the preparation of tubercle antigen.

Experiment 10.—10 c.c. of minced tumour (J.R.S.) were rubbed down to an emulsion in a mortar and washed many times, first with normal saline and then with saline containing 1 per cent. formalin. The liquid was poured off and acetone to a strength of 80 per cent. was then added to the deposit. The fluid was drained away as completely as possible without drying the precipitate and this was then mixed with saline. 1·0 c.c. of the resulting emulsion was

inoculated subcutaneously into the left flank of 20 normal rats, about half of the total of solid matter being used. Three weeks afterwards all the experimental animals and 20 controls were given a test inoculation into the right flank. The results are shown in Fig. 4 and Table VI. It will be seen that the tumours in the experimental animals are smaller than those in the controls, the average volumes of the tumours in the treated animals, reaching 7·56 c.c. as compared with 17·6 c.c. in the untreated.

Experiment 10 has been repeated with some alteration in the technique. In this case the nuclear residue after treatment with formalin was repeatedly washed with 99 per cent. acetone to remove all traces of water. The precipitate was then divided into two parts. An emulsion was made with one of these in normal saline and inoculated into 20 rats. The other was dried in air, reduced to a powder in a mortar, and a vaccine prepared by mixing this powder with saline containing 0·1 per cent. formalin. Twenty rats were treated with this vaccine and given three subcutaneous inoculations at 4 days' intervals.

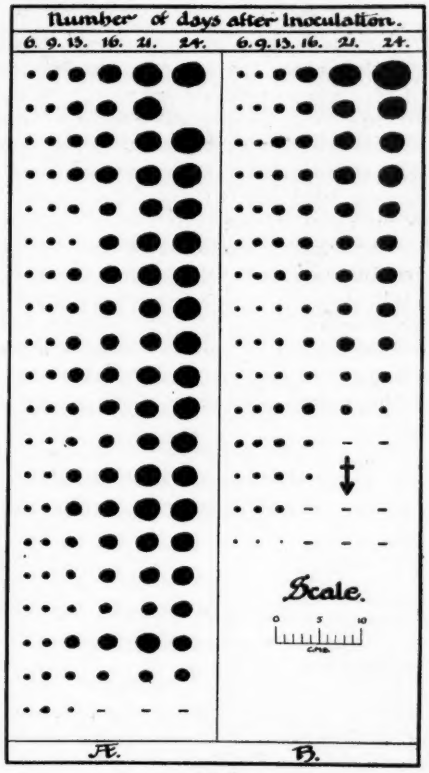

FIG. 4.
A. CONTROLS.
B. EXPERIMENTALS

A subsequent test inoculation showed no immunity in either group of experimental animals.

TABLE VI.

		No. of rats.	Days after inoculation. 6	9	13	16	21	24
Average volume of tumours in c.c.	B. Experimentals	15	0·18	0·28	0·81	1·54	4·17	7·56
	A. Controls	20	0·22	0·53	2·12	4·95	11·2	17·6

The control animals in this table are the same as those in Table II.

We have to thank Dr. J. N. Goldsmith, F.I.C., for many helpful discussions on this problem. Our thanks are due also to Miss D. Pincott for much help in carrying through the experimental investigation, and to Miss D. Clephan for preparing the diagrams.

CONCLUSION.

Evidence has been produced which is confirmatory of the working hypothesis that the antigen causing tumour immunity, in the case of Jensen's rat sarcoma, is a soluble product of the dead tumour-cell formed as a result of enzyme action. The difficulties of detecting and isolating this antigen are increased by the fact that the breaking down of the cell is accompanied by the liberation of a substance which stimulates tumour growth. This body appears in the early stages of autolysis and as far as is known is resistant to enzyme action, and is only slowly eliminated when once absorbed.

Apparently a stimulant to cell growth is given off from the nuclear structures of cells during division and during autolysis. According to Prof. F. G. Hopkins (1923) this stimulant can act in very dilute solutions, and is a growth-promoting catalyst rather than a building material. Clearly its presence, even in minute quantities, may frustrate attempts to detect an antigen. In spite of many negative results we have repeatedly obtained signs of the presence of the antigen in solution, but we have not succeeded in finding evidence of it in any experiment in which the tumour-cells have been mechanically disintegrated. Whether under these circumstances the antigen fails to be formed, or is destroyed by enzyme action or is only masked by the presence of the growth catalyst is at present unknown.

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COLORIMETRIC DETERMINATION OF pH IN PATHOLOGICAL PLASMA BY CULLEN'S METHOD.

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Received for publication November 28th, 1923.

CULLEN (1922) found that the total correction (the difference between the pH found colorimetrically at 20° and the electrometric pH at 37°) in his method of estimating the pH of plasma or serum varied with the species of animal from which the plasma was obtained and was different for plasma and serum. It is probable that this variation is due to differences in protein, quantitative or qualitative. The possibility therefore arises that the correction required for human plasma containing abnormal amounts of protein may differ from that found for normal plasma—a possibility suggested by Cullen, but not further dealt with by him as far as we know.

We have, therefore, compared the pH determined by Cullen's method with that calculated from the Henderson-Hasselbalch ratio in various pathological plasmata in which changes in the proteins were expected.

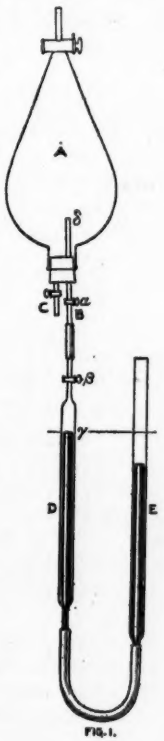

FIG. 1.

METHODS.

(1) *Exposure to CO_2.*— Into a conical separating funnel A (Fig. 1) of 332 c.c. capacity was blown alveolar air from one of us, or, if a higher CO_2 tension was required, CO_2 from a generator. Into this funnel 6 c.c. of blood in Experiments 1 and 2, or 3 c.c. of plasma in the remaining experiments, were introduced from a syringe through the capillary tube C. Mercury was introduced into both capillary tubes, to eliminate dead spaces, and caps put on each. The funnel was then rotated about twice a second in a water-bath at 37° C., the blood or plasma forming a thin layer on the walls of the funnel.

(2) *Estimation of pH and $[BHCO_3]$.*—The funnel was held vertically in a deep water-bath at 37° C. to allow the fluid to collect at the stopper end, taken out, and rapidly dried with a cloth. If blood was used it was withdrawn with a syringe,

the dead spaces of which were filled with oil, and squirted under oil into a centrifuge tube which was surrounded by a water-jacket at 37° C.; the tube was corked, rapidly spun, and the colorimetric pH and [$BHCO_3$] estimated by the methods of Cullen (1922) and Van Slyke (1922). If plasma was used 1 c.c. was first withdrawn with a small syringe and used for pH determination, then the rest of the plasma removed with another syringe and 1 c.c. used for [$BHCO_3$] determination.

(3) *Estimation of CO_2 tension.*—The funnel was cooled in a bucket of water to room temperature, and the pressure at room temperature determined with the apparatus shown in the figure. After connecting B to D with the taps closed (D and the rubber junction being filled with mercury), the taps α and β were opened and E lowered until the mercury in D fell to the mark γ. The difference between the heights of the mercury columns in D and E was then read. The volume $\delta\gamma$ was known, the pressure of the gas in A when expanded to a given volume was determined, and hence the pressure at 37° C. and volume equal to the volume of A less the amount of fluid introduced was calculated.

The actual pH was calculated from the CO_2 tension and [$BHCO_3$] by the formula given by Austin *et al* (1922).

Proteins were estimated by the method of Wu (1922).

DISCUSSION.

In the table are included nineteen consecutive experiments. Only those have been excluded in which some obvious error occurred; it is possible that the CO_2 estimates in Experiments 7 and 8 are incorrect.

EXPERIMENT	PATIENT	DATE	CO_2 TENSION	[$BHCO_3$]	pH CALCD	pH COLOR.	CORRECTION	FIBRINOGEN	ALBUMEN	GLOBULIN	REMARKS
1	—	AUG 21ST 1923	29	20·3	7·49	7·70	0·21	—	—	—	MIXED DIABETIC. BLOOD USED.
2	G.	AUG 23RD 1923	34·1	23·7	7·48	7·73	0·25	—	4·0	1·2	CEREBRAL SYPHILIS. BLOOD USED.
3	—	AUG 30TH 1923	58·7	24·7	7·26	7·50	0·24	0·45	5·35	2·02	MIXED DIABETIC.
4	M.	SEPT 11TH 1923	36·0	28·2	7·53	7·78	0·25	0·25	2·50	1·00	PARENCHYMATOUS NEPHRITIS. LIPÆMIA.
5	M.	SEPT 25TH 1923	33·8	27·0	7·54	7·77	0·23	0·35	2·20	1·75	" "
6	M.	" "	225	29·5	6·76	7·00	0·24	"	"	"	" "
7	P.	SEPT 29TH 1923	67·0	23·4	7·18	7·38	0·20	0·57	3·62	2·90	" "
8	P.	" "	47·3	23·4	7·33	7·60	0·27	"	"	"	" "
9	P.	OCT. 11TH 1923	34·3	11·8	7·17	7·37	0·20	0·55	3·20	1·61	" "
10	P.	" "	34·1	11·8	7·17	7·38	0·21	"	"	"	" "
11	P.	OCT. 24TH 1923	41·9	11·6	7·08	7·30	0·22	0·60	2·86	1·14	" "
12	P.	" "	39·3	11·5	7·10	7·32	0·22	"	"	"	" "
13	RO.	OCT. 20TH 1923	65·3	29·0	7·29	7·52	0·23	0·19	4·41	2·68	ACUTE NEPHRITIS.
14	L.	SEPT. 23RD 1923	38·2	24·0	7·44	7·68	0·24	0·50	2·67	1·94	PARENCHYMATOUS NEPHRITIS. LIPÆMIA.
15	L.	OCT. 29TH 1923	39·9	24·8	7·43	7·66	0·23	0·57	2·35	1·94	" "
16	RA.	OCT. 23RD 1923	33·8	22·7	7·46	7·69	0·23	0·53	2·86	1·14	CHRONIC INTERSTITIAL NEPHRITIS.
17	H.	NOV. 1ST 1923	41·4	28·1	7·47	7·73	0·26	0·40	5·00	1·26	PLEURISY.
18	BA.	OCT. 25TH 1923	37·1	20·0	7·37	7·60	0·23	0·23	3·17	2·14	INFECTIVE ENDOCARDITIS.
19	BI.	NOV. 9TH 1923	36·3	25·6	7·46	7·70	0·26	0·56	3·75	2·82	PYELITIS. PREGNANCY.

In these unselected experiments the average difference between the colorimetric pH and the calculated pH—"the correction"—is 0·23, which is the same as found by Cullen in ten plasmata. The maximum deviations from 0·23 are ± 0·03, unless we include the doubtful Experiment 8, in which it was + 0·04.

The errors which might have occurred in our experiments are—in estimation of CO_2, 1 per cent.; in estimation of $[BHCO_3]$, 2 per cent.; due to variation of temperature during pressure determination, 0·3 per cent. It is not probable that any appreciable loss of CO_2 from the fluid took place while it was being withdrawn from the funnel, as the surface it presented was small, and the reduction of pressure in the funnel during the withdrawal was only 1·8 per cent. with blood and 0·3 per cent. with plasma. So that errors in the calculation of the hydrogen ion concentration may amount to about 3 per cent., which would produce an error in pH of about 0·013.

In the colorimetric estimation there is a possible error of 0·02 in pH—probably less—less, in fact, on some days than on others, and less when the apparent pH was 7·4 or 7·5 than when it was 7·7 or 7·8.

The total possible error was therefore about 0·033 in pH. The corrections found only deviated from 0·23 by more than this in the doubtful Experiment 8, and the average deviation is about half this, *i. e.* 0·015. We may therefore conclude that deviations from the correction 0·23 as we have found are within the limits of error of the methods employed.

The changes in plasma proteins encountered were increase of fibrinogen up to double the normal, reduction of albumen and total protein down to half the normal, and relative and absolute increase of globulin.

The total protein in the majority was decreased and in eleven the fibrinogen was definitely increased. The values for the correction deviated no more from the average in the more extreme cases than in the others, and the average for the whole series was the same as found by Cullen. The method may therefore be used without modification of the correction with such abnormal plasmata.

We consider this conclusion important, as the method of Cullen is an extremely valuable one. Used with Van Slyke's method of estimating the plasma bicarbonate it gives two of the three interdependent variables, pH, bicarbonate, and CO_2 tension, directly on the plasma from a sample of blood. No correction for changes of oxygen saturation are necessary, and there is no risk of acid-formation as there is when blood is equilibrated with an atmosphere of CO_2. Also it requires ordinary laboratory apparatus only and no special proficiency such as is required for determinations of pH in blood or plasma by the hydrogen electrode.

We would lay stress on the necessity of using freshly prepared standards; we have found them fade more rapidly than did Cullen, especially those of higher pH.

We have also found that the stock disodium-hydrogen-phosphate solution does not keep well even in the ice-chest. Such changes in the phosphate solutions are the main source of error; small quantities of stock alkaline phosphate should be made up when required and not kept more than a week. It is therefore convenient to do several determinations on the same or con-

secutive days. When fresh specimens of solid phosphate have to be used their phosphorus content can be checked conveniently by Briggs's (1922) method against a standard which has been checked gravimetrically.

CONCLUSION.

The correction in Cullen's colorimetric method of determining the pH of plasma (the difference between pH found colorimetrically at 20° and the actual pH at 37°) does not change with abnormalities of the plasma proteins.

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VARIATIONS IN THE NUMBER OF BLOOD-PLATES ASSOCIATED WITH A COMMON COLD.

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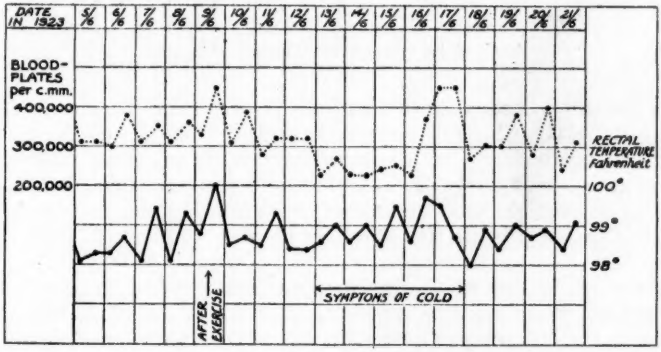
Received for publication November 8th, 1923.

IT has been shown that the disappearance of micro-organisms from the blood-stream into which they have been introduced is preceded by their agglutination (Bull, 1914, 1915, 1916), and further, that this agglutination is brought about by the blood-plates (Delrez and Govaerts, 1918). Cramer, Drew and Mottram (1921–22) have observed the incidence of infective conditions in animals suffering from thrombopenia. From these and other findings it appears that the blood-plates are concerned in the resistance to bacterial infection, and a determination of plates to the site of action would infer at least a temporary fall in their number in the circulating blood whence they are drawn. The following observation would appear to come into line with that hypothesis.

The subject was a case of apparently arrested pulmonary tuberculosis: during a period in which routine daily counts of blood-plates were being made he developed an acute naso-pharyngeal "cold," with the usual symptoms. A technique described by the writer (1923) was employed to make counts between 9 and 10 a.m., and between 5 and 6 p.m. At these times of the day certain other variations which are being studied can be excluded. Each

figure recorded is the mean of two counts from different preparations made at the same time, and, with few exceptions, not differing by more than 10 per cent. The rectal temperature was noted at the same time.

Many observations (unpublished) have shown that there is a general relation between the temperature and the number of plates in circulation; this is exemplified in the chart by a daily variation which is notably exaggerated on 9/6/23, when there occurred a rise of temperature due to the taking of exercise. The average plate-count of this subject in his normal condition lay in the region of 340,000 per c.mm., but, as shown in the chart,



the number fell during the "cold" to about 240,000 per c.mm. until the terminal stages of the "cold," when a rise above the normal average occurred and was then followed by a resumption of the normal level. It may be noted that during this mild but acute infection there was little disturbance of temperature, and the associated plate-curve is to be contrasted with that found in chronic febrile phthisis, for in that condition very large plate-counts, *e. g.* 700,000 per c.mm., have been repeatedly recorded.

The observations were carried on during the tenure of a grant from the Medical Research Council.

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ON THE REPUTED CHEMICAL STIMULATION OF ANTI-BODY PRODUCTION.

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Received for publication January 23rd, 1924.

AT the present moment there seems to be a certain amount of confusion concerning the relationship of non-specific protein therapy and the non-specific stimulation of antibodies. In all probability, however, these phenomena are distinct, as it is quite likely that non-specific protein therapy has, in most instances, no relation to true antibody formation. In the present article it is proposed to deal only with the question of the non-specific stimulation of antibodies.

The attention of one of the authors was first drawn to this subject about twelve years ago when working on the therapeutic effects of salvarsan.

Soon after the discovery of chemo-therapeutic remedies various suggestions were put forward to explain their mode of action, one being that these substances increased the antibody content of the serum. For instance, experiments were brought forward claiming that "606" could increase the amount of agglutinins for infections other than spirochætal (Nicolas, Gourmont and Gate, 1912, and others). In a more recent communication Walker (1920) gives particulars of instances where he found a rise in the agglutination titre after salvarsan. He states that "the rise may amount to 200 or 300 per cent. or even more."

The cure of a congenital syphilitic infant by its mother's milk after treatment of the mother by "606" was said to be due to excess of syphilitic antibodies in the milk. Observations made in conjunction with Dr. Fildes on this point failed to demonstrate excess of antibodies and chemical examination showed the presence of minute quantities of arsenic (McIntosh and Fildes, 1911). It was therefore thought probable that the repeated administration of these small doses of arsenic produced the therapeutic effect.

The only direct evidence that "606" injections had any relation to antibody production was elicited in the treatment of relapsing fever in rats. Injections of "606" into the infected animals causes a rapid disappearance of the spirochætes, and this disappearance is coincident with the appearance of antibodies (agglutinins) in the serum (McIntosh, 1910). As this might quite well be an indirect result of the cure of the disease an experiment was devised where the question of actual cure of an infection was avoided.

In this experiment rabbits were first immunized against sheep red corpuscles by repeated injections, and then some days after the last injection,

when the amboceptor titre was relatively constant, injections of salvarsan were given. The control injection consisted of normal saline and the volume in each case was 50 c.c. The titration of the amboceptor strength of the sera was performed by the method adopted for the Wassermann test. In this, the serum is diluted 1 in 1000 in saline and quantities from 0·1 c.c. to 0·9 c.c. distributed into a series of test-tubes; then 0·1 c.c. of a one-fold dilution of guinea-pig complement is added and this volume made up to 1·0 c.c. with normal saline, and finally 0·5 c.c. of a 5 per cent. suspension of red cells is added. Hæmolysis is judged to be complete at the end of 2 hours at 37° C.

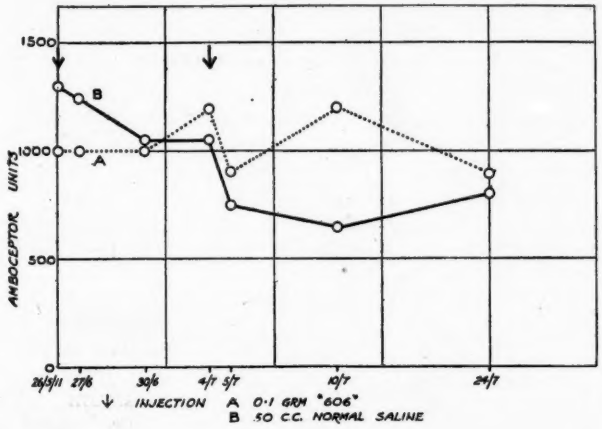

CHART No. 1.

It will be seen from Chart No. 1 that the control animal gave a slight fall in the amboceptor titre after each injection of normal saline. After the first injection of "606" the titre of the serum remained constant for two days and then rose from 1000 to 1200 units; a second injection at this date produced a slight fall which 24 hours later gave place to a slight rise. Somewhat similar results were obtained in another experiment. These observations were not pursued, as it was considered that any stimulating effects which "606" possessed in the matter of increasing antibody production was too slight to be of any practical importance.

The subject of the non-specific stimulation of antibody production has again been brought into prominence by the recent publication of Madsen (1923) and his collaborators. These writers claim that antibody production can be stimulated successfully by a number of simple chemical substances such as manganous chloride, beryllium chloride, etc. In some instances an increase of over 1000 per cent. was obtained.

In view of these results, and in spite of our earlier failures, it was resolved to test the effects of some of the chemicals used by Madsen and his workers. In this series of experiments rabbits immunized against sheep red corpuscles and against *B. typhosus* were used. Most of the tests were made with beryllium

chloride—the compound found by Madsen to be the most efficacious. For the preparation of the chemical solutions used we are indebted to our research chemist, Mr. W. Lawson, M.Sc., A.I.C.

The rabbits were immunized against the sheep corpuscles by receiving 4 injections at intervals of 24 hours of 1·0 c.c. of washed and deposited cells.

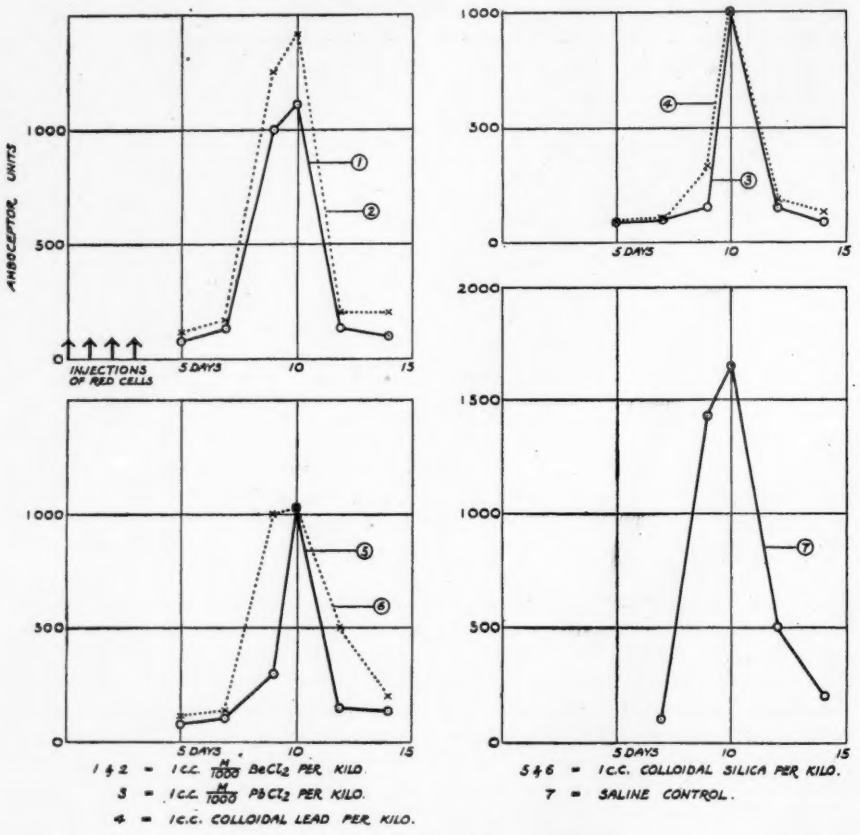

CHART No. 2.

The immunization curves were quite typical in their rise and fall. To make the test severe the chemical substances were injected towards the lower part of the fall as is shown in the charts.

In Chart No. 2, curve Nos. 1 and 2, the rabbits were injected with beryllium chloride; in Nos. 3 and 4, lead chloride and colloidal lead; in Nos. 5 and 6, colloidal silica, and No. 7 was used as control. The colloidal solutions corresponded roughly to M/1000 solutions of the elements contained.

A study of these curves shows that, given towards the end of the immunization curve, the chemicals tested had no effect on the antibody content of the serum.

In another experiment where rabbits were immunized against *B. typhosus* and then injected with beryllium chloride similar negative results were obtained. Chart No. 3 gives the curve of one of the animals.

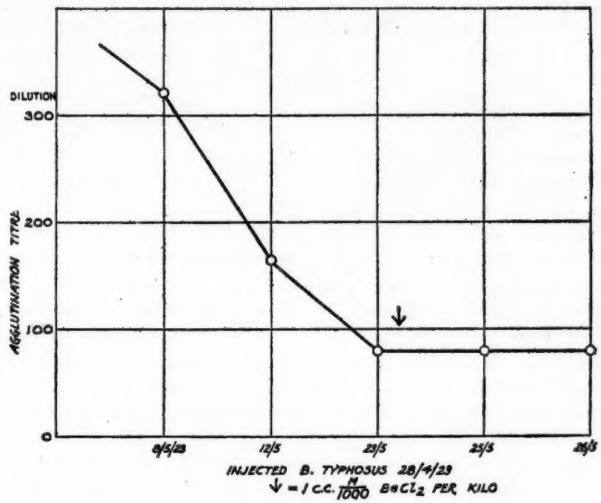

CHART NO. 3.

DISCUSSION.

From the experiments detailed above, no evidence was deduced that injections of simple chemical substances produce any significant rise in the antibody content of the serum of immunized animals. The quantities of the substances injected were necessarily small owing to their toxic nature. Where the chemical employed had been used by the Danish workers corresponding doses were given.

The slight rise observed in Chart No. 1 after "606" is not considered significant, as in our opinion nothing under 100 per cent. should be counted, when in the dilution the volume is halved each time. These experiments confirm our earlier opinion that simple chemical substances have little, if any, power of increasing antibody production. The tests were all severe in that the injections were made at a time when the immunity curve was falling and less response was expected.

The divergence between our findings and those of Madsen and his collaborators requires comment. This cannot be explained on the fact that horses and goats were used more than rabbits. Also it is hardly likely that the methods employed in the titration of sera are at fault, because they are the

methods universally used at the present time. There is just a remote possibility, however, that from some cause or another (such as infection, vitamine deficiency or repeated bleedings) the animals were not strictly normal. Under such conditions the chemical injected might act as a tonic, thus indirectly acting as a stimulant of antibodies. That the condition of affairs was not quite normal when the tests were made is borne out by an analysis of the chart showing the average production of diphtheria toxin and antitoxin at the Danish State Serum Institute from 1903 to 1922. From the year 1903 till 1914–15 the toxin and antitoxin production curves ran quite parallel, with the antitoxin curve on the top. From 1914–15 till the present a reversal of the curves occur; at the former date a fall in the strength of the toxin is observed, with a corresponding fall in the antitoxin titre. Such a falling off of the toxin titre is not uncommon at times in serum institutes, and is explicable on the use of unsuitable media or with the falling off of toxin production by the bacillus. Then the toxin production curve gradually rose till about 1919–20 it had reached its old level. Arguing from the first part of the curve there should have been a corresponding rise in the antitoxin production, but this is delayed, and only resumes the old relation in 1921–22, when the chemical stimulants are used. In the later part of that year a higher average titre is shown, following the application of these chemical substances. Madsen himself states that "diphtheria horses" whose antitoxin concentration has been constantly decreasing show a considerable rise after manganous chloride. But we cannot consider such "exhausted" horses as normal animals.

Though this may explain the results obtained with antitoxic horses we are still without an explanation in the case of rabbits, if we are prepared to accept rises in titre which may almost be regarded as remaining within the limits of experimental error. Moreover, on theoretical grounds, it seems unlikely that an injection of a simple chemical substance will stimulate the antitoxin value of the serum to any great extent. If this were so then a single injection of such a substance might rapidly bring about a cure in an ordinary case of diphtheria.

CONCLUSIONS.

No experimental evidence was obtained that any significant increase in the antibody content of the sera of immunized animals can be produced by the injection of simple chemical substances.

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THE SOURCE AND CHARACTERISTICS OF CERTAIN CULTURES SENSITIVE TO BACTERIOPHAGE.

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Received for publication January 12th, 1924.

Of the remarkable facts recorded in connection with the Twort-d'Herelle phenomenon the two following possess a special interest, which is enhanced by their apparent inconsistency:

(1) The extreme specificity of some samples of bacteriophage, *e. g.* one strain of *B. coli*, *B. typhosus* or *B. dysenteriæ* is acted on, and no other.

(2) In marked contrast with (1) is the wide range of cultures over which a single sample of bacteriophage may be active; for example, certain strains of *B. dysenteriæ* (Shiga), *B. typhosus* and *B. coli* may be sensitive to the same phage.

These two characters appear often to be shown by the same specimen of phage. D'Herelle (1922) mentions that some strains of *B. typhosus* are naturally resistant to a given phage which acts on others of the same species, although the resistant strains may be lysed by another phage from a different source. He also describes filtrates of phage which will lyse some stock cultures of *B. dysenteriæ* and *B. typhosus*, but not another more recently isolated strain of the former organism. He states that *B. dysenteriæ* (Shiga) is a homogeneous species from the point of view of the bacteriophage, *i. e.* that a phage which is active for one strain is active for all. He does not, however, appear to have found this to be literally true, for he says that in the case of *B. dysenteriæ*, as well as of *B. typhosus*, repeated culturing on agar lowers the resistance of freshly isolated strains to the bacteriophage. He also states that resistant strains of *B. dysenteriæ* and other members of the coli-typhoid group occur and are inagglutinable.

Object of the Present Research.

In the first place experiments with bacteriophage were undertaken to find out whether certain variants which had been obtained from *B. dysenteriæ* (Shiga) strain 550 were uniform in their relation to the phage. These variants had been isolated in the course of an examination of the limits of variability in different directions of Shiga's bacillus. Subsequently, other strains were examined with the object of producing similar varieties.

The Sources of the Strains of B. dysenteriæ and of the Bacteriophage which were used.

All the observations here recorded were made with cultures of Shiga's bacillus. The bacteriophage which was used throughout was kindly given to the writer by Miss Lepper, who had originally isolated it by filtration from the urine of a case of pyelitis infected with *B. coli*, and had increased it by passage in cultures of the same strain of *B. coli*. It was used for her experiments on the action of bacteriophage (Lepper, 1923).

The cultures of Shiga's bacillus have been obtained from the National Collection of Type Cultures, London. The strains were first plated on agar and the stock cultures kept for experiments were grown from single colonies. All the stock cultures formed acid from glucose, but not from lactose, cane-sugar, mannite or dulcite, did not form indole, and agglutinated with a serum prepared with other strains of Shiga's bacillus.

Six strains have been used, viz. 550 (both smooth and rough variants), 151, 152, "H," "B" and "D."

Strain 550 was originally isolated in Italy in 1918. In 1920 the present writer segregated from the parent strain two variants, "smooth" and "rough," which were described by him (Arkwright, 1921), and were subsequently kept and subcultured separately in the National Collection until November, 1922.

The "smooth" form was characterized by turbid growth in broth, stability of emulsions in salt solution, and a coarse type of agglutination with specific serum, whilst the "rough" form grew with a deposit in broth, leaving the medium clear, agglutinated "spontaneously" in salt solution (NaCl, 0·85 per cent.), and formed very small clumps with agglutinating serum (muddy type of agglutination). The special agglutinins for the two forms were, moreover, to a great extent distinct, as indicated by absorption experiments and by immunizing rabbits with the two forms separately. In November, 1922, the cultures of the two forms had retained their special characteristics in all respects.

The other five strains when received in August, 1923, were all of the smooth form as to character of growth in broth, stability of emulsions, and type of specific agglutination.

METHODS.

Cultures of varying ages in broth or in 10 per cent. peptone water were plated out on agar, and colonies of different appearance were selected and inoculated into broth. These variants were again plated from time to time to see whether they remained true to type, and when fairly permanent were taken into stock and subcultured every seven to ten days. In selecting colonies, especial attention was given to obtaining "rough" colonies which formed a deposit in broth, leaving the supernatant clear, but colonies of the opposite tendency were also sought, so as to obtain where possible extremes in this respect from the same plate.

The use of 10 per cent. peptone water cultures as a source of variants was suggested by De Kruif's statement that with this medium he obtained a

larger proportion of "granular" variants (De Kruif, 1921). The medium has not, however, been used sufficiently by the present writer to establish the fact that it possesses special advantages.

All the variants which have been recorded were found to give the typical sugar reactions and to agglutinate with specific serum, though not always in as high a dilution as the parent strain.

The cultures were tested for sensitiveness or resistance to the bacteriophage by making dilutions in broth of the phage in test-tubes, and inoculating each with a loop of a young broth culture.

These were incubated at 37° C. and subcultured to agar slopes after two and a half to three hours and again after five hours. The broth and agar tubes were again incubated and examined next day for evidence of phage action. The dilutions of phage most frequently used were about 1 in 50 and 1 in 125,000. A larger number of dilutions was often used and subcultures were also made at shorter intervals, but the above method was found usually to supply the information required.

The bacteriophage as originally received was a filtrate of a broth culture made by inoculating a dilution of phage with *B. coli*. Later, batches were made by inoculating a dilution of phage with a sensitive culture of "R3," which was a sensitive variant of strain 550 and will be described later. The resulting clear, or nearly clear, lysed cultures have not been filtered, but have been freed from living bacteria by the addition of chloroform and incubation at 37° C., till they yielded no growth when subcultured to agar. The titre of the bacteriophage, *i. e.* the highest dilution in which definite phage action on strain 550 "R3" could be detected, has always been about 10^{-7} or 10^{-8} when the tests have been made is described above.

In making the foregoing experiments it has been recognized that attempts to demonstrate the presence or absence of bacteriophage action are liable to error if precautions are not taken to find the proper quantitative relations of phage and bacteria for the special case (see Lepper, 1923[1]), and to examine the cultures at a suitable time after the mixture of phage and culture has been made.

No strain has, therefore, been described as resistant unless at least two dilutions of phage have been used, and subcultures have been made to agar in the manner detailed. Evidence of sensitiveness has not been accepted unless definite "nibbled" or "moth-eaten" colonies or *tâches vierges* have been obtained. In cases of doubt the experiment has been repeated with variation of the intervals between inoculation and subculture to agar, and a conclusive result has always been obtained.

RESULTS.

In none of the cultures of original strains or variants has it been found possible to demonstrate typical lytic action without the addition of bacteriophage from an outside source.

All the original strains have been resistant to phage when examined with the probable exception of the "rough" variant, "R" of Strain 550, which had been previously selected as a variant and which failed to show evidence of sensibility at the first test.

The results obtained with the individual strains will now be given *seriatim*.

Strain 550.—Search for variants both of the "smooth" and the "rough" form by plating out old broth cultures was begun in November, 1922, and by the 10th February, 1923, ten minor variants had been isolated. Twelve cultures consisting of the original "smooth" and "rough" forms and the ten variants were inoculated into tubes of the original bacteriophage diluted 1 in 10 with broth. After incubation for five hours subcultures to agar were made and all the tubes incubated at 37° C. On the following day phage action could be detected in only one agar tube, which had been inoculated with a variant, "R3" derived from the "rough" form.

Some days later a more thorough test for sensitiveness with a sample of phage which had been twice "passaged" with "R3" showed that two more of the cultures were sensitive—(1) the original "rough" form, "R," and (2) a "rough" variant, "SV," derived from the "smooth" form. The original "smooth" form and its "smooth" derivatives remained resistant.

The three sensitive varieties derived from strain 550 were by no means exactly alike in other respects besides their agglutinability by neutral salts; in particular "SV" grew on agar in large opaque colonies and formed a very viscous emulsion in water, "R" formed very small colonies on agar and the emulsion was little or not at all viscous, and both of these emulsified well in distilled water, whereas "R3" grew in very small colonies and was emulsified with great difficulty, since the growth when washed off the surface of agar remained in coherent scaly flakes, unless special methods of breaking them up were used. A further difference was that while "SV" always formed a deposit in broth, leaving the supernatant quite clear, broth cultures of "R3" and sometimes of "R" were slightly turbid for the first day or two, though later they cleared with the formation of a deposit. In salt solution (NaCl 0·85 per cent.), however, all three sensitive variants agglutinated, "R3" even in 0·42 per cent. NaCl.

Strain "H."—After cultures of this strain had been plated several times and a number of colonies examined which all proved to be resistant, a sensitive variant was obtained as follows: A culture in 10 per cent. peptone water 10 days old was plated on agar and a "rough" colony picked off to broth. When the resulting culture had grown for nine days it was replated and a "rough" colony again selected. This gave a culture which proved highly sensitive when tested with phage. "Smooth" colonies of "H" obtained at the same time were quite resistant.

Strain "D."—This strain, too, yielded a sensitive variant but less readily than the last. A culture in 10 per cent. peptone water 61 days old was plated and a colony picked off to broth and allowed to grow for 34 days. On plating the last, both sensitive and resistant "rough" colonies were obtained. These were tested with a sample of phage which had been three times passaged with 550 "R3."

Strain "B."—From this strain also a sensitive variant was obtained. A subculture in 10 per cent. peptone water from the original stock was plated on agar on October 8th, 1923, when it was 51 days old. Almost all the resulting colonies were smooth, but one colony of different appearance inoculated into broth formed an almost clear culture with a deposit of growth. When this

was 35 days old, plating on agar gave "smooth" and "rough" colonies. One of these "rough" colonies after 21 days in broth on plating produced a number of "rough" colonies of different kinds as to size and appearance. Some were very small and did not grow when picked off to broth, while others of identical appearance inoculated directly on to agar slopes formed good cultures. These latter, when subcultured by a large inoculum to broth, made a slightly turbid culture with a small amount of scattered deposit. These very small colonies on the plate proved to be a variant which was highly sensitive to phage and gave only sensitive colonies when replated. The larger colonies grew readily in broth and proved to be resistant to phage.

Strains 151 *and* 152.—From these two strains no sensitive cultures have been obtained so far. Cultures of strain 151 only produced "smooth" colonies when plated in the first two months during which it was under examination; later "rough" variants occurred, but none proved to be sensitive.

Strain 152 has never been found to produce "rough" variants, but colonies of very different size and appearance have been seen on plates, showing that variants were being produced; none, however, have proved sensitive to bacteriophage.

Remarks on the Production of Variants.

1. Some strains have yielded variants much more readily than others, *i.e.* it has not always been necessary to wait till cultures have become very old in order to obtain "rough" or other definite variants on plating.

2. The first variation usually seen in cultures has been a change in opacity and size of the colonies and slight irregularity in outline or granularity.

3. In older cultures or on replating old cultures of selected colonies a great variation in the size of colonies has often been noticed, and sometimes cultures of repeatedly selected small colonies have always yielded a few large colonies in addition to the majority of small colonies. When large and small colonies are both present it may happen that the small colonies lie in notches in the larger colonies, which partly encircle but do not touch them. This appearance suggests that there is present an excessive sensitiveness to the natural inhibitory action of neighbouring colonies, or conversely, that an excess of inhibitory action is being manifested. A similar appearance is often seen in agar cultures made from mixtures of phage and sensitive variants.

4. Small colonies have sometimes been obtained on plates especially from strains 550 and "B" which could be subcultured to agar but not to broth, though sometimes growth was obtained in glucose peptone water. The resemblance of this behaviour to similar effects seen in subcultures from mixtures to bacteriophage and Shiga's bacillus is referred to again below.

DISCUSSION OF THE RESULTS.

1. *Spontaneous Variation in Relation to Sensitiveness to Bacteriophage.*

Sensitive variants have been obtained from four out of the six resistant strains of Shiga's bacillus which have been examined, and have remained highly sensitive for as long as they have been under observation, *i.e.* for at least some weeks, but in most cases for two or three months.

It has been known since the early work of D'Herelle, and particularly from the writings of Bordet and Gratia, that after the action of the phage on a sensitive strain both sensitive and resistant races can sometimes be isolated from the resulting culture after partial lysis has taken place.

Also Gratia (1921), by subculturing from a part of an old agar culture of a sensitive strain of *B. coli* which was visibly different from the rest of the culture, obtained a race which was more resistant than the parent form, but apparently not entirely refractory, to his bacteriophage. From the same original strain he also obtained a race of enhanced sensibility. In both instances the culture had not been in contact with the phage before the variants were obtained.

So far as I know, however, evidence has not hitherto been brought forward to show that without the addition of phage a number of variants can be isolated from a completely resistant strain, some of which are sensitive while others are resistant.

2. *Correlation of Sensitiveness and Agglutination by Neutral Salts.*

In the case of all the sensitive variants dealt with in this paper there has been a definite association of the two characters of sensitiveness and agglutinability by salt. This relation has not been quite absolute; moreover, in the two spontaneously agglutinable variants of strain 550 "R" and "R3" there has been a slight reversion towards the "smooth" form during the last few months. The reversion has been far from complete and the emulsions are still agglutinated by 0·85 per cent. NaCl, although the broth cultures are distinctly turbid with only a slight deposit during the first few days after inoculation. While many "rough" variants have given no proof of sensitiveness, "smooth" variants have always been fully resistant. The definite but incomplete correlation of "roughness" and sensitiveness seems to be on a *par* with the relation of other variable characters of bacteria in respect of which persistent variants occur. The fact that purity of such variants is often hard to obtain may partly account for the incomplete way in which such associated characters are correlated. It often happens that though two characters are notably associated they nevertheless appear to be partly independent, and the observation that they often coincide amounts to little more than overlapping. Thus in recorded observations on the association of such characters as "spontaneous agglutination" (roughness) on the one hand with a "muddy" type of specific serum agglutination, agglutinability by "rough" specific agglutinins and sensitiveness to the bacteriophage on the other, complete correlation has not been established, but there is a considerable overlap.

In the same way specific agglutination and sugar reactions in the typhoid-coli-dysentery group of bacteria are not always perfectly correlated. The same may be said for variation which affects other characters and suggests that these are often due to separate hereditary factors.

3. *Variations in Cultures induced by the Action of Bacteriophage.*

The experiments with sensitive races and bacteriophage have given the opportunity of observing the varied appearances associated with the Twort-d'Herelle phenomenon.

When a culture in broth has been inoculated with bacteriophage and clearing has occurred, a subculture to broth may show no sign of growth, but a subculture to agar may yield many colonies. Again, when the characteristic action of bacteriophage has taken place, either primarily on the surface of agar or in broth which is subsequently transplanted to agar, one of several appearances may present themselves:

(*a*) There may be no visible growth.

(*b*) No growth may appear, if the subculture is made during the first hours of incubation, but later subcultures may give a growth of large opaque colonies.

(*c*) After initial absence of growth a number of very small colonies may appear later if incubation is prolonged.

(*d*) No growth may occur except of a very few colonies at the top of the slope where the agar is thinnest and driest.

(*e*) In subcultures made at a certain stage, varying with the strength of the phage, the striking picture is seen of a confluent growth of the bacterium with empty clear round spots—the *tâches vierges* or "colonies of bacteriophage" of d'Herelle. These *tâches* may later be occupied by minute colonies resembling those seen at later stages on some, at first completely blank, agar slopes described in (*c*).

(*f*) At other times almost confluent growth may occur at first, but on further incubation some groups of colonies may be lysed and form *tâches*.

(*g*) Appearances which are perhaps even more remarkable are seen when the proportions of bacteria and phage are just correctly adjusted in broth and a subculture to agar is made at the most suitable time—usually 3 to 5 hours after the commencement of incubation. In these cultures a number of colonies of very different aspects appear at about the same time: (1) Large colonies with very irregular nibbled margins; (2) very small round colonies, some of which are placed in semicircular notches in larger colonies; (3) colonies of intermediate sizes and of varying shapes and degrees of opacity, which are distributed very irregularly over the surface of the agar, leaving blank spaces which are probably formed by merged *tâches*. On picking off single colonies from such a culture the following results may be obtained:—(1) No growth may occur in broth which may be found sterile after incubation, but a good growth may occur if the colonies are picked off to agar slopes; (2) growth from the colonies subcultured may take place readily either in broth or agar and the resulting cultures may prove to be—(*a*) resistant to the phage and also may produce more of the lytic agent, *i.e.* may be "lysogenic"; (*b*) resistant but not lysogenic; (*c*) sensitive to the phage but unlike the parent culture in size and form of colony.

EXPLANATORY HYPOTHESIS.

These varied phenomena under the influence of the phage were in part described by Bordet and Ciuca (1920), and have been repeatedly observed by the present writer. They have been explained in different ways:

(1) D'Herelle, who believes the bacteriophage to be a living organism parasitic on the bacteria, thinks that the varied characters of the colonies are

due to the selection by the destructive action of the phage of the more resistant individuals whose "natural immunity" enables them to survive. He apparently considers that the resistant forms are pre-existent in the culture, but also describes an "acquired immunity," the result of the phage action.

(2) Gratia (1921) also maintained that the different races, which could be isolated after phage action, were pre-existing in the culture and of different degrees of resistance, but he differed from d'Herelle in thinking the selecting agent to be of the nature of an enzyme rather than a living organism.

(3) Bordet and Ciuca (1920) early in the controversy maintained that the "bacteriophage" was of the nature of an enzyme, and brought forward experiments to support their view. They considered the mode of action to be that the "phage" entered the growing bacteria and so affected them as to produce an hereditary alteration or vitiation of their metabolism, as a result of which fresh lytic substance was produced by them. Bordet summed up the nature of the lytic factor as "contagious and hereditary." He has lately (Bordet, 1923) again expressed the view that the essential action is a disturbance of the metabolism of the bacteria giving prominence to the autolytic function.

(4) Eastwood (1923) has put forward detailed hypotheses to account for the results produced by the phage, which he maintains are due to a non-living enzyme-like agent. He may be said to be in sympathy with Bordet's main view, since he attaches prime importance to the initiation of variation in the bacteria by the transmissible agent, and he emphasizes the condition of phage action, which was originally pointed out by d'Herelle, that the bacteria must be in active division in order that the phage may produce its characteristic effects.

(5) Lepper, (1923[1, 2]) has shown that the lyticprocess can be divided into different stages, has measured the rate of action of the phage in different dilutions, and brought forward evidence that adsorption of the lytic agent on to the bacteria plays an important part as a stage in the process.

SUMMARY OF RESULTS AND DEDUCTIONS REGARDING THE NATURE OF BACTERIOPHAGE ACTION.

I. One very conspicuous effect of the action of bacteriophage on a suitable culture of bacteria is the variation produced in the bacteria. This variation appears too great to be due to a mere selection of pre-existent forms, which normally only occur very rarely, except in selected cultures. The variation is so sudden that it must be due to change in the bacteria within a very few generations. In old cultures without the addition of phage, variation also occurs which has certain points of resemblance to the changes produced by the action of phage, but only takes place very slowly or at rare intervals. This similarity is shown particularly by the occurrence in both cases of colonies and cultures with the following characters:

(1) Colonies which are sensitive to the phage, and others which, like the parent culture, are quite resistant.

(2) Small colonies which appear to have an especially inhibitory or lytic action on larger colonies present on the same agar surface. The small colonies lie in concave notches in the larger colonies.

(3) Colonies which, when transplanted to broth, do not grow, but form a good culture if inoculated on the surface of agar.

(4) Cultures in broth are often obtained after phage action has taken place, which, when subcultured to broth, make no growth, the broth remaining sterile, but when inoculated on to agar form good growth. Broth cultures of "spontaneous" variants sometimes behave in the same way, *e. g.* some variants of strain "B."

The inability of certain cultures to grow in broth whilst they are viable when inoculated on to agar is apparently due to the comparative dryness of the agar giving an advantage to the bacteria, whilst the liquid medium favours lytic action. No doubt the same explanation accounts for the appearance of growth only in the dry upper part of agar slopes when lysed cultures are subcultured to agar. Doerr and Berger (1923) have recorded experiments bearing on this phenomenon which they maintain show that gelatine and, to a less degree, agar, inhibit the action of the bacteriophage.

The behaviour of all these variants indicates the action in the cultures of an excess of the bacteriolytic function in some or all of the bacteria present, and suggests that the phenomenon is of the same kind in the case of the spontaneous variants and of those induced by bacteriophage action.

A similar conclusion is indicated by the experiments of Otto, Munter and Winkler (1922). These writers claim to have obtained highly active bacteriophage with considerable regularity by repeated "passage" *in vitro* of normal strains through heated filtrates of homologous cultures.

It seems unlikely that the sensitive and other variants procured from normal cultures which suggest bacteriophage action are due to previous contamination with phage which has been lying dormant, since this would necessitate the hypothesis that phage is frequently, if not always, present in cultures of organisms of this class. For instance, in the present investigation, sensitive variants have been obtained from four out of six strains of Shiga's bacillus which have been examined. In some instances this result has only been obtained after repeated plating and selection of colonies, suggesting that the remaining two strains would probably yield similar sensitive variants on further examination.

II. The active agent which is transmitted to the fresh culture when phage is added to a sensitive strain is a stimulus to variation, especially in a certain direction. It is a substance which disturbs the balance of the growing bacteria in the direction of autolysis. There does not, however, seem to be sufficient evidence to show that the transmissible agent is itself the "lytic factor," though it may be so. In order to prove this it would be necessary to demonstrate lysis of bacteria in a culture or emulsion within a space of time, after the addition of phage, too short for division to have taken place.

This conclusion may seem to be in contradiction to the evidence that strong bacteriophage appears to act like a disinfectant and rapidly to kill bacteria added to it (Lepper, 1923[2]). It is, however, only maintained here that bacteria after treatment with phage do not die till after growth has begun, as is known to happen when weaker concentrations of phage are inoculated with culture.

Absence of growth after subculture does not prove that lysis or death has

already occurred at the time of subculture. The defect in growth is probably due to autolysis of the new generation as soon as it has been produced.

If this is the case the name "transmissible lytic agent" is not strictly applicable.

III. The action of bacteriophage on a young culture has been regarded as consisting of two factors—inhibition and lysis. It does not seem to be clearly established that inhibition, except as the expression of autolysis of newly-formed bacteria, actually occurs.

IV. These considerations make it highly probable that the essential factor in the "bacteriophage phenomenon" is the stimulus to variation which occurs to a small extent "spontaneously" in many old cultures, and when present in an enhanced degree produces so large a number of autolytic and lysogenic individuals that "bacteriophage action" becomes obvious. The agent which stimulates to variation is produced in the act of autolysis, and in this way is effected its transmission. This view is not incompatible with those of Bordet and Ciuca (1920), Gratia (1921) and Bordet (1923).

V. In order to form a consistent picture of the action of bacteriophage, it is necessary to attempt to explain the relation to each other of individual bacteria or colonies which are sensitive, lysogenic or resistant respectively. The relationship appears to be as follows: Sensitive cultures consist of individuals which react to the transmissible stimulus by producing—(1) autolytic individuals, *i. e.* bacteria which lyse themselves and reproduce the stimulating agent; (2) resistant individuals which are not affected by the transmissible stimulus and only give rise to similar resistant forms; (3) resistant (lysogenic) forms which are not noticeably affected by the phage, but give rise besides the resistant forms to the sensitive form and to autolytic individuals, which latter lyse themselves and reproduce the transmissible agent.

The difference between autolytic and sensitive individuals is probably one of degree, the former rapidly undergoing lysis spontaneously, the latter requiring the stimulus of the transmissible agent.

Resistant and sensitive forms no doubt vary in their power of resisting or reacting to the transmissible stimulus.

VI. The nature of the bacteriophage action is not less important if the conclusion is accepted that the phenomenon is due to a one-sided exaggeration of the natural metabolism of the bacteria. The transmissibility of an active agent and its simulation of a highly resistant filterable virus form a very striking train of occurrences, which perhaps indicate a principle applicable to some infectious processes in higher organisms.

CONCLUSIONS.

1. Variants sensitive to "bacteriophage" arise "spontaneously" in old normal cultures from resistant strains.

2. The sensitive variants found during these experiments have always been "rough," *i.e.* agglutinated by salt solution (NaCl, 0·85 per cent.), but all "rough" variants have not been sensitive, though all the "smooth" cultures examined have been resistant to phage.

3. Other spontaneous variants of normal cultures, *e.g.* small colonies

inhibiting the growth of large, and colonies, which will not form a culture in broth but grow well on agar, occur, and suggest spontaneous autolytic (" bacteriophage ") action.

4. Production of variants by phage action is a very conspicuous feature of the Twort-d'Herelle phenomenon.

5. The variants obtained by phage action and without the addition of phage are very similar.

6. The relation (sensitiveness) of special "spontaneous" variants to phage supports the view that phage action is due to an exaggeration of normal autolytic action.

7. The transmissible factor is a stimulus which produces variants, especially such as produce an excess of autolysis.

8. The similarity of the variants which occur with and without phage suggests the identity of the stimulus evoking these variants in both cases.

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THE LIVER AND CERTAIN OTHER TISSUES IN RELATION TO ACQUIRED IMMUNITY TOWARDS JENSEN'S RAT SARCOMA.

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Received for publication November 2nd, 1923.

IN previous papers we (Lazarus-Barlow and Parry, 1923[1 and 2]) have considered intra-cerebral and intra-splenic immunization with Jensen's rat sarcoma, and have shown that immunization by either of these routes is more effective than that by subcutaneous inoculation. In the present paper the work is extended upon identical lines to the liver, and comparisons are instituted between the various routes of immunization that have been examined.

TABLE I.—*Comparison of Intra-hepatic and Subcutaneous Immunization with Jensen's Rat Sarcoma upon the Development of a Subcutaneous Graft. A = Intra-hepatic, B = Subcutaneous Immunization.*

Exp.	Immunizing graft. Character.	Immunizing graft. Frequency of local takes. A. %	Immunizing graft. Frequency of local takes. B. %	Test tumour on 10th day. A. Takes. % of control.	Test tumour on 10th day. A. Volume. % of control.	Test tumour on 10th day. B. Takes. % of control.	Test tumour on 10th day. B. Volume. % of control.	Healthy and tumour-free survivals. A. %	Healthy and tumour-free survivals. B. %
1	Fresh	60	71	47	12	53	16	40	29
2	Fresh	41	75	39	35	73	95	59	25
1	Frozen and thawed	37	67	42	35	83	51	47	25
2	Frozen and thawed	0	0	121	176	133	169	44	5
1	Irradiated	17	24	33	11	76	30	67	29
2	Irradiated	0	31	83	106	100	177	47	19

Series of twenty normal rats were immunized intra-hepatically with grafts of either fresh, rapidly growing tumour, or tumour altered by repeated freezing or thawing, or by exposure to a heavy dose of X rays.* Seven days later the animals were inoculated subcutaneously with a test graft of a fresh rapidly growing tumour. Measurements of the subcutaneous grafts were made at 2-day intervals, and each experiment was continued for five weeks from the immunizing and four weeks from the test inoculation, when the surviving

* The irradiation was under standard conditions as in the previous papers and was kindly carried out for us by Miss Gladwys Scott. The freezing and thawing was repeated eight times as in the experiments on spleen.

animals were killed and examined for growth macroscopically and microscopically. The degree of effect in each series of rats was estimated by (1) the percentage of "takes" of the test graft on the 10th day, (2) the amount of tumour present at the site of subcutaneous test inoculation on the 10th day, and (3) the ultimate survival of healthy tumour-free rats. Similar series were immunized subcutaneously for purposes of comparison and numerous controls were made at each stage of the experiments. The experiment was repeated, and the values obtained in both experiments are set forth in Table I.

From these experiments we conclude that intra-hepatic immunization leads to a higher degree of immunity than subcutaneous immunization.

THE RELATIVE DEGREES OF IMMUNITY INDUCED BY THE INTRA-CEREBRAL INTRA-SPLENIC, INTRA-HEPATIC AND SUBCUTANEOUS ROUTES.

If the values given in this and the preceding papers be summarized and collated an indication can be obtained of the relative degree of immunity conferred by each of the immunization routes employed. These are given in Tables II, III and IV.

TABLE II.—*Takes of Test Tumour on 10th Day after Immunization by Various Routes compared with Corresponding Subcutaneous Immunization. All Values are Percentages of Controls.*

Immunizing graft.		Brain.	Sub-cutaneous.		Spleen.	Sub-cutaneous.		Liver.	Sub-cutaneous.
Fresh	(i)	59	79	(i)	32	69	(i)	47	53
	(ii)	76	69	(ii)	29	24	(ii)	39	73
	(iii)	72	24		—	—		—	—
Frozen and thawed		—	—	(i)	72	89	(i)	42	83
		—	—		—	—	(ii)	121	133
Irradiated	(i)	75	50	(i)	73	66	(i)	33	76
	(ii)	76	66	(ii)	64	44	(ii)	83	100
	(iii)	70	44		—	—		—	—

TABLE III.—*Volumes of Test Tumour on 10th Day after Immunization by Various Routes compared with Corresponding Subcutaneous Immunization. All Values are Percentages of Controls.*

Immunizing graft.		Brain.	Sub-cutaneous.		Spleen.	Sub-cutaneous.		Liver.	Sub-cutaneous.
Fresh	(i)	62	65	(i)	26	56	(i)	12	16
	(ii)	129	56	(ii)	11	25	(ii)	35	95
	(iii)	153	25		—	—		—	—
Frozen and thawed		—	—	(i)	55	89	(i)	35	51
		—	—		—	—	(ii)	176	169
Irradiated	(i)	62	23	(i)	75	107	(i)	11	30
	(ii)	114	107	(ii)	62	32	(ii)	106	177
	(iii)	68	32		—	—		—	—

TABLE IV.—*Percentage of Survivals of Healthy and Tumour-free Rats after Immunization by Various Routes compared with Survivals after Corresponding Subcutaneous Immunization.*

Immunizing graft.		Brain.	Subcutaneous.		Spleen.	Subcutaneous.		Liver.	Subcutaneous.
Fresh	(i)	19	0	(i)	23	0	(i)	40	29
	(ii)	0	0	(ii)	74	10	(ii)	59	25
	(iii)	17	10		—	—		—	—
Frozen and		—	—	(i)	29	0	(i)	47	25
thawed		—	—		—	—	(ii)	44	5
Irradiated	(i)	29	5	(i)	40	42	(i)	67	29
	(ii)	20	42	(ii)	42	25	(ii)	47	19
	(iii)	33	25		—	—		—	—

Inasmuch as the results in the foregoing tables were obtained from a number of experiments, the intra-hepatic, intra-splenic and subcutaneous routes of immunization were considered in a single simultaneous experiment upon three batches of twenty animals. This experiment was a very severe test. For a fresh tumour was used for immunization, and this on subcutaneous inoculation took in 100 per cent. of cases and yielded on the 10th day a mean volume of 293 c.mm., while the tumour used for test purposes also took in 100 per cent. of cases and yielded on the 10th day a mean volume of 560 c.mm. The results of this experiment are given in Table V, and a graphic representation of the behaviour of the test inoculation is given in the figure.

TABLE V.—*Results of Simultaneous Intra-splenic, Intra-hepatic and Subcutaneous Immunization with Fresh Tumour upon the Development of Subcutaneous Test-graft.*

Intra-splenic immunization.			Intra-hepatic immunization.			Subcutaneous immunization.		
Test tumour on 10th day.		Survivals.	Test tumour on 10th day.		Survivals.	Test tumour on 10th day.		Survivals.
Takes % of control.	Vol. % of control.	%.	Takes % of control.	Vol. % of control.	%.	Takes % of control.	Vol. % of control.	%.
53	16	12	76	40	6	84	46	0

Thus the results of this experiment accord with those already detailed.

From all these experiments we conclude that when fresh or frozen and thawed tumour is used for immunization the resulting immunity is greater with intra-splenic, intra-hepatic or intra-cerebral immunization than with subcutaneous, and of these intra-splenic immunization gives the best results.

With intra-cerebral inoculation the early phenomena of immunity are less marked than with subcutaneous immunization, but retrogression of the test tumours occurs, and the ultimate survival of healthy and tumour-free animals is greater.

With intra-hepatic and intra-splenic inoculation both the early phenomena of immunity and the survival of healthy and tumour-free animals are more marked than with subcutaneous immunization.

Splenic and hepatic immunization with fresh and with irradiated tumour present certain interesting differences. Whereas fresh tumour induces the

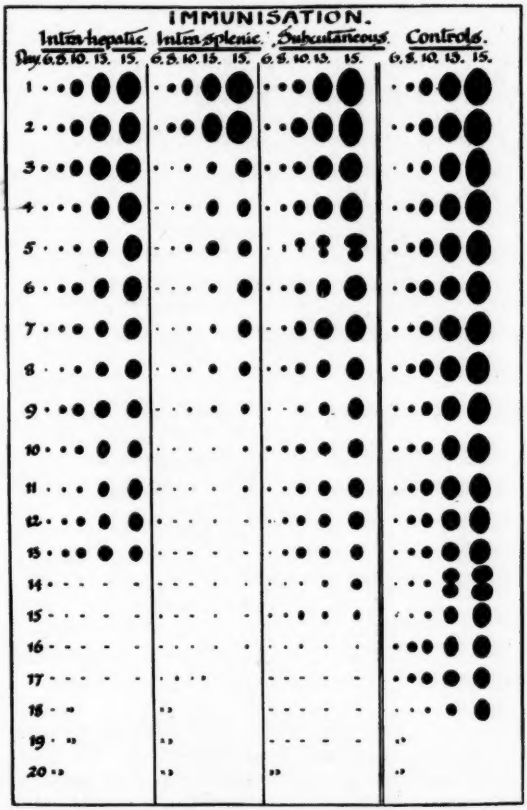

FIG. 1.—Development of the test (subcutaneous) tumour after immunization by various routes (simultaneous experiment).

highest degree of immunity when inoculated into spleen, irradiated material induces the highest degree of immunity when inoculated into liver. This fact suggested that processes concerned with immunity initiated in the fresh tumour-cells by the spleen were elaborated by the liver. In other words, that the spleen was concerned with formation of antigen from the fresh tumour-cells, and the liver, receiving this antigen by way of the splenic vein, was concerned with the production of antibody.

Evidence upon this point was sought by an endeavour to divert the splenic blood from the portal into the systemic circulation. In spite of numerous attempts this direct experiment failed. It was easy to herniate the spleen through the abdominal muscles and new formation of superficial blood-vessels occurred, but subsequent ligature of the splenic pedicle was followed invariably after one or two days by complete necrosis of the spleen and death of the animal.

That a special relation obtains between spleen and irradiated tumour has been pointed out in a previous paper (*loc. cit.*), where it was shown that heavily irradiated tumour-tissue, when inoculated into the spleen, is associated with a far greater mortality of the animals from no readily assignable cause than when such material is inoculated into other tissues.

PERCENTAGE FREQUENCY OF LOCAL TAKES IN SUSCEPTIBLE ANIMALS.

These values for the entire series of experiments described in this and the preceding papers are given in Table VI. The data consist in the final results of the initial or immunizing inoculation determined, if necessary, by microscopic examination. Since this preceded the second or test subcutaneous inoculation by seven days it is considered that the latter did not seriously affect the result.

TABLE VI.—*Percentage Frequency of Local Takes.*

Immunizing graft.		Brain.	Subcutaneous.		Spleen.	Subcutaneous.		Liver.	Subcutaneous.
Fresh	(i)	81	100	(i)	59	100	(i)	60	71
	(ii)	100	100	(ii)	21	90	(ii)	41	75
	(iii)	83	90		—	—		—	—
Frozen and thawed		—	—	(i)	0	30	(i)	37	67
		—	—		—	—	(ii)	0	0
Irradiated	(i)	43	43	(i)	0	26	(i)	17	24
	(ii)	11	26	(ii)	0	10	(ii)	0	31
	(iii)	11	10		—	—		—	—

In the simultaneous liver, spleen and subcutaneous immunization experiment to which reference has been made above (p. 36), the local takes in spleen were 77 per cent., in liver 82 per cent., and in subcutaneous tissue 100 per cent. In addition, the spleen in two cases contained small completely degenerated masses of growth; in liver and subcutaneous tissue all the growths were large and vigorous.

From these experiments we conclude that in respect of local resistance to growth of a graft of Jensen's rat sarcoma, whether fresh, or altered by repeated freezing and thawing, or by heavy irradiation, the order of the tissues examined is as follows: Spleen (greatest), liver, brain, subcutaneous tissue (least).

All available evidence tends to show that the production of acquired immunity by means of tumour-cells is bound up with changes occurring in the still living but dying or degenerating cells. Thus the greatest percentage of

immunity is seen in batches of animals that have been inoculated with a variety of tumour that takes in a relatively small percentage of cases, grows slowly, and frequently undergoes spontaneous retrogression. Apparently it is because local resistance in the spleen is greatest that intra-splenic inoculation of grafts is associated with the greatest production of general immunity.

THE GENERAL CHARACTER OF THE ACQUIRED IMMUNITY.

In view of Levaditi and Nicolau's (1923) observations upon the neurotropic and dermotropic varieties of immunity with vaccinia, it was thought possible that reliance upon subcutaneous inoculation of fresh tumour-grafts for test purposes might be insufficient for a comparison of immunity induced by (*a*) hepatic, and (*b*) subcutaneous routes. Thirty-nine rats immunized by the hepatic route and six immunized by the subcutaneous route, all of which had proved immune to subcutaneous test grafts on one or two occasions, were inoculated with a fresh tumour-graft into the kidney. In all but one instance the renal graft failed.

Similarly 3 rats that had been immunized by the subcutaneous route and had proved immune to subcutaneous test inoculation were subjected to intra-hepatic test inoculation. In no instance did growth take place.

And finally, a series of 25 rats immunized by the subcutaneous route and immune to subcutaneous test inoculation proved, in every instance, to be also immune to a further simultaneous intra-cerebral and subcutaneous test inoculation with fresh rapidly growing tumour.

It may therefore be concluded that the immunity induced by either the hepatic or the subcutaneous route is general in a wide sense of the term.

CONCLUSIONS.

We conclude that in the case of Jensen's rat sarcoma and the normal rat—

1. The degree of immunity acquired after inoculation of tumour-graft, whether fresh or altered by repeated freezing and thawing, differs according to the route of immunization being greatest with intra-splenic and least with subcutaneous. Intra-cerebral and intra-hepatic immunization occupy an intermediate position.

2. When heavily irradiated tumour is used as the immunizing agent, the intra-hepatic route is the most effective of the four considered.

3. Variations in the degree of local resistance to growth of an implanted immunizing graft are to be associated with variations in the degree of acquired immunity, and probably play a fundamental part in its production.

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THE REPRODUCTION OF BACTERIOPHAGE WHEN THE SENSITIVE ORGANISM IS GROWN IN A SYNTHETIC MEDIUM.

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Received for publication January 11th, 1924.

PRESENT STATE OF KNOWLEDGE AS TO THE REPRODUCTION OF BACTERIOPHAGE.

It has been shown by a number of workers that bacteriophage is reproduced only in the presence of growing organisms.

Doerr (1922), using the Appelman-Werthemann method, made repeated observations on the titre of the lysin and the number of organisms present during the course of the reproduction of a bacteriophage. He found that a definite relationship existed between the growth of the organisms and the increase in the lysin. Using a dilution of bacteriophage which was a hundred times the minimal amount (eL = 2),* the following sequence of events took place: During the period of bacterial lag the titre of the lysin was stationary, while during the period of logarithmic growth of the organisms the titre of the lysin increased rapidly, reaching a maximum in about four hours. The organisms grew normally for three hours, reached a maximum at four hours, and were then rapidly lysed.

Meuli (1923) found that with a minimal dose of lysin the organisms increased up to the end of five and a half hours and were then lysed. The titre of the lysin began to increase about the third hour and reached a maximum at the end of seven hours.

The bacteriophage which I have been using behaves in the manner described above. A dilution of 1/100 million (eL = 2) in peptone water inoculated with a drop of a culture of the sensitive bacillus and then incubated has returned to its original strength by the end of five hours. Lysis of the culture begins about the end of the fourth hour and may be complete in half an hour.

Using the minimal dose of 1/10,000 million (eL = 0) the reproduction of the lysin is complete in seven hours.

It is of interest to note that with few exceptions the titres of the bacteriophages described in the literature whether for organisms of the dysentery or coliform groups appear to be of the same order of magnitude.

Maitland (1922) was led to believe from his experiments that for the production of bacteriophage three factors are necessary—the lysin, a growing sensitive organism, and an accessory substance which is present in peptone, serum and potato extract.

As the *B. coli* with which I have been working grows well on a synthetic medium in which the source of nitrogen is an ammonium salt and the carbon is supplied by glucose, it seemed of interest to find out if it were possible to obtain reproduction of the bacteriophage in the absence of protein.

* Werthemann's (1922) method of expressing the titre of the lysin is based on the convention employed for the hydrogen-ion concentration of solutions. If the highest dilution of the first tube of the series (containing 1 c.c. of the lysin and 9 c.c. of broth) which shows lysis of the culture be 1/1,000,000, or 10^{-5}, then the "Lysinexponent," eL = 5; if 1/10,000 million or 10^{-9}, then eL = 9. When the highest effective dilution has been reached it is expressed for convenience as eL = 0. This apparent anomaly is due to the fact that in the first tube of the series the lysin has been diluted 1/10. In the text, for the sake of clearness, the actual dilution of the lysin used in the experiments is given as well as the lysin exponent.

EXPERIMENTS WITH SYNTHETIC MEDIUM.

EXPERIMENT I.—*To determine the rate of growth of the bacillus in the synthetic medium.*

Composition of the medium used:—The medium suggested by Ayres (1918) for the isolation of *B. coli* from water was employed:

Ammonium phosphate 0·4 grm.
Sodium di-hydrogen phosphate 0·2 grm.
Glucose 1·0 grm.
Distilled water 100 c.c.

The medium was sterilized by steaming on three successive days. The pH was 6·8 to 6·9.

A tube containing 5 c.c. of this medium was inoculated with 0·02 c.c. of a peptone-water culture (2 per cent. peptone in ·5 per cent. sodium chloride) of the *B. coli* and incubated. Samples were removed at intervals and plated. The rate of proliferation as compared with the growth of the same organism in peptone water is shown in Chart I.

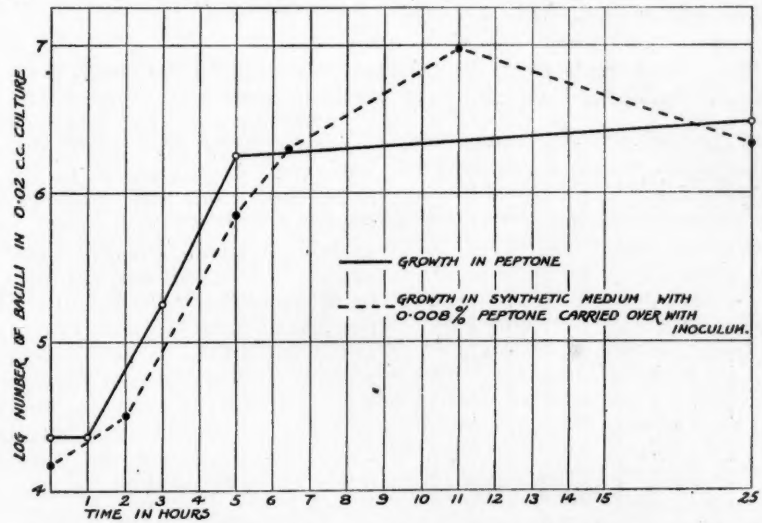
LOG NUMBER OF BACILLI IN 0·02 C.C. CULTURE
7
6
5
4
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 25
TIME IN HOURS
GROWTH IN PEPTONE
GROWTH IN SYNTHETIC MEDIUM WITH 0·008% PEPTONE CARRIED OVER WITH INOCULUM.

CHART I. TO COMPARE GROWTH OF B. COLI IN SYNTHETIC MEDIUM WITH GROWTH IN PEPTONE.

It will be seen that the rate of growth is appreciably slower in the synthetic medium but that a larger number of organisms is present after seven hours. This is probably due to the sugar in the medium.

EXPERIMENT II.—*To determine the influence of various amounts of peptone incorporated with the synthetic medium on—(α) the reproduction of a bacteriophage, (β) the rate of growth of the sensitive bacillus.*

A series of tubes containing various amounts of synthetic medium, peptone and bacteriophage as shown in Table 1 were set up and inoculated with 0·02 c.c. of a peptone-water culture of the bacillus. The tubes were incubated.

The rate of growth of the *B. coli* in the control tubes containing no lysin is shown in Chart II.

TABLE 1.—*To show the Influence of Various Amounts of Peptone added to the Synthetic Medium, on the Reproduction of Bacteriophage.*

Number of tube.	Composition of medium. Synthetic.	Peptone.	Final % of peptone.	Dose of lysin in 0·5 c.c. saline. eL.	Dilution.	Number of bacilli at end of 5 hours in ·02 c.c.	Time of heating cultures.	Titre of lysin in heated cultures.
1	4·5 c.c.	0·02 c.c.	0·008	eL=2	1/100 million	Numerous	5½ hours	eL=5
2	4·4 „	0·12 „	0·048	„	„	163	„	eL=7
3	4·3 „	0·22 „	0·088	„	„	22	„	„
4	4·0 „	0·52 „	0·208	„	„	0	„	„
5	0·0 „	4·5 „	1·8	„	„	5	„	eL=9
6	4·4 „	0·12 „	0·048	eL=0	1/10,000 million	Numerous	6¾ „	eL=7
7	4·0 „	0·52 „	0·208	„	„	3	„	eL=9
8	0·0 „	4·5 „	1·8	„	„	0	„	„
9	5·0 „	0·02 „	0·008	None				
10	4·9 „	0·12 „	0·048	„				
11	0·0 „	5 „	2	„				

Plates were made from the cultures containing bacteriophage after five hours' incubation, and the number of organisms remaining viable estimated.

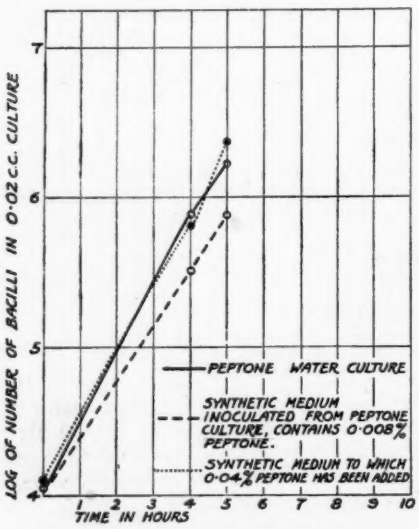
LOG OF NUMBER OF BACILLI IN 0·02 C.C. CULTURE
7
6
5
4
1 2 3 4 5 6 7 8 9 10
TIME IN HOURS
PEPTONE WATER CULTURE
SYNTHETIC MEDIUM INOCULATED FROM PEPTONE CULTURE, CONTAINS 0·008% PEPTONE.
SYNTHETIC MEDIUM TO WHICH 0·04% PEPTONE HAS BEEN ADDED

CHART II. TO SHOW THE EFFECT OF THE ADDITION OF SMALL AMOUNTS OF PEPTONE TO THE SYNTHETIC MEDIUM ON THE GROWTH OF B. COLI.

After five and a half hours' growth the cultures containing 100 doses of lysin were heated at 57° C. for one hour, those containing 10 doses were sterilized after six and three quarter hours' incubation. The titre of the lysin in these heated cultures was estimated by Appelman's method, the turbidity in the various dilutions tested being noted at the end of five hours and subcultures made from them on agar slopes. The highest dilution which on agar showed evidence of bacteriophage was taken as the end-point (see last column of Table 1). This dilution had generally shown a partial clearing of the bacillary growth.

Results.—(1) Bacteriophage is reproduced in a synthetic medium containing no proteid except that carried over with the culture inoculated.

(2) The lysin thus produced has a much lower titre than the original strain, the end-point being 1/million as compared with 1/10,000 million in peptone water.

(3) A small amount of peptone—0·04 per cent.—has a considerable effect on the titre of the lysin produced, increasing it a hundred-fold.

(4) The presence of 0·2 per cent. peptone may suffice to enable the bacteriophage to reach its full titre (Tube 7).

(5) It will be seen in Chart II that 0·04 per cent. peptone added to the synthetic medium was sufficient to raise the rate of growth of *B. coli* to that which occurs in peptone water.

Conclusions.—(1) The titre of a bacteriophage reproduced by incubation with an organism growing in a synthetic medium is 1/10,000 less than that of the original bacteriophage.

(2) The difference in the rate of growth of the sensitive bacillus in the two media does not appear to be sufficient to account for this phenomenon. The number of bacilli present per drop at the end of five hours in a peptone-water culture is about 1,700,000, as compared with 790,000 in the synthetic medium. *It does not seem probable that a difference of two to one in the possible number of bacilli present should make a difference of ten thousand to one in the amount of lysin formed.* Furthermore, the addition of 0·04 per cent. peptone in the synthetic medium raises the rate of growth of the bacillus to that which is found in peptone water, and yet the titre of the lysin produced in such a medium is a hundred-fold less than that of the original bacteriophage.

EXPERIMENT III.—*To determine whether bacteriophage can be transmitted in series in the synthetic medium.*

The sample of bacteriophage obtained from Tube 1 in Exp. III having a titre of eL=5 (1/1,000,000) was passaged five times in the synthetic medium inoculated with a drop of a peptone-water culture. The final dilution was $(1/10,000)^5$. If the original amount of lysin had been diluted, 1/100 would have been the limit of its activity. The bacteriophage thus produced had a titre of eL=3 as against eL=9 of the peptone control. When incubated with the sensitive bacillus it caused clearing of the culture at the end of five hours, but this was transient, as in twenty-four hours the tubes were as cloudy as the control without lysin. With the original lysin, although there was some cloudiness due to the development of resistant bacilli, the tubes remained less cloudy than the control. Subcultures made on agar at the end of five hours from the lysed cultures showed inhibition of growth, nibbled colonies or the presence of "plages." These appearances on agar were, however, not nearly so stable as those obtained from the stock bacteriophage, as the "plages" were often rapidly overgrown by the surrounding culture even at room temperature. The lysin when incubated in a peptone-water culture gave very definite clearing of the growth followed by cloudiness. Agar subcultures made at five hours when the culture was clear showed thick confluent growths without evidence of bacteriophage. Passage in peptone five times did not restore the original strength of the lysin.

The transient effect produced by this bacteriophage resembles that described by Doerr (1923) as occurring in a strain which he isolated from the fæces of a hen. This he found had a titre of eL=5 to eL=6. When incubated with a sensitive culture the tubes showed clearing in between six and nine hours. Reproduction to maximal titre was found in the highest dilution which showed this temporary lysis.

EXPERIMENT IV.—*To determine whether bacteriophage can be transmitted in series in complete absence of peptone.*

The lysin obtained in Exp. II, Tube 1, was again used. It was incubated in synthetic medium with a culture of the *B. coli* which had been subcultured daily on the synthetic medium for a month. The amount of peptone carried over from the first subculture was therefore negligible.

The growth curve of the bacillus in this medium is shown in Chart III. The period of bacterial lag is longer than in peptone or synthetic medium inoculated from a peptone culture.

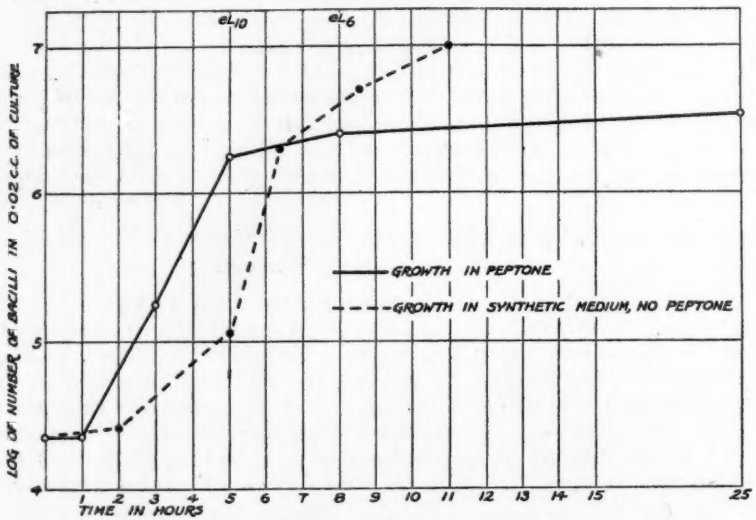
eL10
eL6
LOG OF NUMBER OF BACILLI IN 0·02 C.C. OF CULTURE
7
6
5
4
GROWTH IN PEPTONE
GROWTH IN SYNTHETIC MEDIUM, NO PEPTONE
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 25
TIME IN HOURS

CHART III. TO COMPARE GROWTH OF B.COLI IN SYNTHETIC MEDIUM WITH GROWTH IN PEPTONE. NO PEPTONE CARRIED OVER WITH INOCULUM.

After five hours' growth the tubes containing the culture and lysin were heated for an hour at 57°C. and the titre of the bacteriophage determined.

The result was negative, all trace of lysin being lost at the third passage.

Starting again and watching the effect of a dose of the stock bacteriophage eL=3 (1/10 million) on the synthetic culture over a longer period, it was noticed that although clearing of the culture had begun by the end of five hours *the maximum effect was not produced for eight hours.* Samples of culture were therefore removed at six, eight, ten and eleven and a half hours, sterilized at 57°C. and their bacteriophage content titrated.

Results.—(1) No increase in lysin was found in the six-hour sample.

(2) The eight- to eleven-hour samples had a titre eL=5 (1/1,000,000). This was transmitted in series three times in synthetic medium, the final dilution being a million million times that of the 1/10 million dilution of bacteriophage originally used. There was therefore definite evidence that the

lysin had increased in the synthetic medium, and the dilution was such as to reduce any proteid added with the bacteriophage to a negligible amount.

(3) In both synthetic medium and in peptone the same transient effect was produced on the *B. coli* as had already been noted in Exp. III.

(4) Three passage cultures in peptone did not restore the lysin to its original strength.

Conclusions.—(1) Bacteriophage can be transmitted in series in a synthetic medium.

(2) The lysin thus obtained is considerably less active than the original strain.

(3) Its activity is not restored by several passages in a peptone culture.

DISCUSSION OF RESULTS.

The cause of the failure of the bacteriophage to return to its original strength when incubated with a culture growing on a synthetic medium is not clear. It may be that the bacteriophage is a virus and that it has become attenuated by growth under unfavourable conditions. On the other hand, if bacteriophage results from the products of bacterial growth, these may be sufficiently different in the synthetic medium to account for the alteration in the strength of the lysin produced.

Against both these views is the fact that the bacteriophage does not return to its original strength when incubated with a culture in peptone.

The delay which occurs in the reproduction of the lysin in synthetic medium, no increase in the titre being found at the end of six hours, although considerable proliferation of the bacilli has occurred, supports Maitland's view that some accessory substance is necessary for the production of bacteriophage.

In the case of synthetic medium this may be supplied by bacterial proteid or its degeneration products, hence the absence of reproduction of the lysin until a later stage in bacterial growth, when numbers of dead bacilli must be present.

The experiments with varying amounts of peptone added to the synthetic medium show that with a good growth of the sensitive bacillus a very small amount of proteid, peptone 0·04 per cent., has a pronounced effect on the titre of the lysin produced.

Some experiments to determine whether the activity of the modified bacteriophage could be restored by contact with broth or the products of bacterial growth proved negative. An accessory substance if such exists would probably have to be present at the time of formation of the bacteriophage.

Miss Chick suggested that the peptone might act in virtue of the alteration it produces in the surface tension of the synthetic medium. Experiments in which the surface tension has been altered by other means have so far proved negative.

It was thought that the results obtained might be due to the development of a particularly resistant type of bacillus. The immediate lethal effect of a 1/500 dilution of the stock bacteriophage was therefore tested on a culture which had been subcultured daily in the synthetic medium for a week. It

acted on a three hours old culture of this organism at the same rate as it did on a peptone culture of the same age. There does not appear, therefore, to be a higher proportion of resistant organisms present in the synthetic medium.

Further investigations seem, therefore, desirable.

SUMMARY.

(1) A bacteriophage can be reproduced and transmitted in series by incubation with a sensitive organism growing in a synthetic medium.

(2) The bacteriophage thus obtained is much less active than the sample from which it has been derived. It produces transient clearing of peptone-water cultures, and appears to be without effect so far as one can judge from appearances on the agar subcultures made from these.

(3) This modified lysin is not restored to its original strength by five passages in peptone cultures.

(4) Peptone is not essential for the reproduction of the bacteriophage, but even in small amounts (*e.g.* 0·04 per cent.), has a marked effect on the titre of the lysin produced.

(5) The increased bacterial growth caused by the addition of peptone to the synthetic medium does not appear to be sufficient to explain the much more marked effect which such addition has on the reproduction of bacteriophage.

(6) The experiments recorded support Maitland's view that some accessory substance is necessary for the complete reproduction of bacteriophage.

I am very grateful for the laboratory facilities accorded me at the Lister Institute which have made this work possible.

I am greatly indebted to Dr. Ledingham and Miss Chick for much helpful advice and criticism, and to Dr. Mary Cowan for her kind assistance with subcultures.

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